

Biotechnology in Agriculture Series, No. 29

Biotechnology of Fruit and Nut Crops



Edited by
R.E. Litz



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Edited by

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Contents

Contributors	ix
Preface	xiii
Dedication	xv
Oded Reuveni	xv
Uri Lavi and Emanuel (Emi) Lahav	
Dirk R. Vuylsteke	xvii
Emile A.G. Frison	
Introduction	xix
John S. (Pat) Heslop-Harrison	
1 Actinidiaceae	1
1.1 <i>Actinidia</i> spp. Kiwifruit	2
M. Margarida Oliveira and Lena G. Fraser	
2 Anacardiaceae	29
2.1 <i>Anacardium occidentale</i> Cashew	30
Rajani Nadgauda, Subramanian Jayasankar and Richard E. Litz	
2.2 <i>Mangifera indica</i> Mango	40
Richard E. Litz and Miguel A. Gómez-Lim	
2.3 <i>Pistacia vera</i> Pistachio	62
Ahmet Onay, Isikalan Cigdem and Adiyaman Filiz	
3 Annonaceae	73
3.1 <i>Annona</i> spp. Atemoya, Cherimoya, Soursop and Sugar Apple	74
Carlos Lopez Encina	
4 Arecaceae	89
4.1 <i>Cocos nucifera</i> Coconut	90
Valérie Hocher, Jean-Luc Verdeil and Bernard Malaurie	
4.2 <i>Elaeis guineensis</i> Oil Palm	113
Alain Rival and Ghulam Kadir A. Parveez	
4.3 <i>Phoenix dactylifera</i> Date Palm	144
Kenza Loutfi and Ismail El Hadrami	
5 Bromeliaceae	157
5.1 <i>Ananas comosus</i> Pineapple	158
M.K. Smith, H.-L. Ko, G.M. Sanewski and J.R. Botella	

6	<i>Caricaceae</i>	173
6.1	<i>Carica papaya</i> Papaya Maureen M.M. Fitch	174
7	<i>Clusiaceae</i>	209
7.1	<i>Garcinia mangostana</i> Mangosteen Sompong Te-chato and Mongkol Lim	211
8	<i>Ericaceae</i>	221
8.1	<i>Vaccinium</i> spp. Blueberry Lisa J. Rowland and Freddi A. Hammerschlag	222
8.2	<i>Vaccinium</i> spp. Cranberry Brent H. McCown and Eric L. Zeldin	247
9	<i>Fagaceae</i>	263
9.1	<i>Castanea</i> spp. Chestnut F. Javier Viéitez and Scott A. Merkle	265
10	<i>Juglandaceae</i>	297
10.1	<i>Carya illinoensis</i> Pecan Wagner Vendrame and Hazel Wetzstein	298
10.2	<i>Juglans regia</i> Walnut Abhaya Dandekar, Chuck Leslie and Gale McGranahan	307
11	<i>Lauraceae</i>	325
11.1	<i>Persea americana</i> Avocado Richard E. Litz, Witjaksono, Simon Raharjo, Darda Efendi, Fernando Pliego-Alfaro and A. Barceló-Muñoz	326
12	<i>Moraceae</i>	349
12.1	<i>Ficus carica</i> Fig, <i>Artocarpus</i> spp. Jackfruit and Breadfruit and <i>Morus</i> spp. Mulberry Vishwas A. Bapat and Minal Mhatre	350
13	<i>Musaceae</i>	365
13.1	<i>Musa</i> spp. Banana and Plantain M.K. Smith, S.D. Hamill, D.K. Becker and J.L. Dale	366
14	<i>Myrtaceae</i>	393
14.1	<i>Psidium guajava</i> Guava Uma Jaiswal and V.S. Jaiswal	394
15	<i>Oleaceae</i>	403
15.1	<i>Olea europea</i> Olive Eddo Rugini and Luciana Baldoni	404
16	<i>Oxalidaceae</i>	429
16.1	<i>Averrhoa carambola</i> Carambola Richard E. Litz and John L. Griffis, Jr	430
17	<i>Passifloraceae</i>	435
17.1	<i>Passiflora</i> spp. Passionfruit Maria Lucia C. Vieira and Monalisa Sampaio Carneiro	436

18	<i>Rosaceae</i>	455
18.1	<i>Fragaria</i> Strawberry <i>Julie Graham</i>	456
18.2	<i>Malus</i> × <i>domestica</i> Apple <i>Susan K. Brown and Kevin E. Maloney</i>	475
18.3	<i>Prunus</i> spp. Almond, Apricot, Cherry, Nectarine, Peach and Plum <i>C. Srinivasan, Isabel M.G. Padilla and Ralph Scorza</i>	512
18.4	<i>Pyrus</i> spp. Pear and <i>Cydonia</i> spp. Quince <i>Elisabeth Chevreau and Richard Bell</i>	543
18.5	<i>Rubus</i> spp. Cane Fruit <i>Robert M. Skirvin, Sergio Motoike, Matthew Coyner and M.A. Norton</i>	566
19	<i>Rutaceae</i>	583
19.1	<i>Citrus</i> Grapefruit, Lemon, Lime, Orange, etc. <i>Gloria A. Moore, Jude W. Grosser and Frederick G. Gmitter</i>	584
20	<i>Sapindaceae</i>	627
20.1	<i>Dimocarpus longan</i> Longan and <i>Litchi chinensis</i> Litchi <i>Richard E. Litz and Simon Raharjo</i>	628
21	<i>Sterculiaceae</i>	637
21.1	<i>Theobroma cacao</i> Cacao <i>Antonio Figueira and Laurence Alemanno</i>	639
22	<i>Vitaceae</i>	671
22.1	<i>Vitis</i> spp. Grape <i>Dennis J. Gray, S. Jayasankar and Z. Li</i>	672
Index		707

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Preface

More than a decade has passed since Dr Freddi Hammerschlag and I co-edited *Biotechnology of Perennial Fruit Crops*. Unlike many biotechnology reference books, this text remained largely relevant for several years and many of the chapters are still almost state-of-the-art for some under-researched species. That book was organized to provide intensive coverage of the major fruit crops and the well established and emerging biotechnologies that were impacting studies with these plants.

Biotechnology of Perennial Fruit Crops went out of print a few years ago and the publisher and I decided that it should be replaced rather than revised. Our reasoning was that more fruit species should be covered to reflect the increased activity with 'minor' crops, and that nut crops should be included. We also considered that the section of the book that described biotechnologies should be eliminated, since this information is now widely available. Another major change has involved the organization of the book according to plant family, which would seem to be more rational than other arbitrary groupings, i.e. fruit and nut crops, major and minor crops and tropical/subtropical and temperate species.

One of the striking similarities in almost every review chapter in this book is that almost all of our standard fruit and nut cultivars have arisen outside the agency of conventional plant breeding. There are many straightforward reasons for this: the long juvenile period of most of these species, the lack of genetic diversity in plant collections, the resistance of consumers to change, etc. For these and other reasons, it is possible that biotechnology will have a much more profound impact upon improvement of perennial fruit and nut trees than any other group of commodities.

Notwithstanding the current anxiety in some countries about the safety of transgenic food, this technology will almost certainly be utilized in other countries to increase yields and reduce production costs. Consumers and societies can either reject or accept this technology; indeed, many past advances in technology were rejected for reasons that defy rational explanation. Regardless of the outcome, the older biotechnologies, including somatic hybridization, *in vitro* mutagenesis and selection, ploidy manipulation, etc., are only now being exploited for many of the perennial fruit and nut crop species discussed in this book. Genomics and the application of marker-assisted selection to conventional breeding of fruit and nut crops will be important tools for the medium and long term.

I am indebted to the authors of this book and to several other people, who helped me in various ways. I would like to acknowledge the technical assistance of Pamela Moon. I would also like to thank Tom Gradziel, Bart Panis, Ebrahim Firoozabady, Jim Hancock, Courtney Weber, Ray Schnell, Robert Knight Jr, Simon Raharjo, Witjaksono and Subramanian Jayasankar for their careful review of manuscripts. Special thanks go to Jorge Peña.

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Dedication

Since the publication of *Biotechnology of Perennial Fruit Crops*, we have noted the loss of three leaders in our field, all of whom are noted for their significant contribution to banana cell and tissue culture: Franticek Novak, Oded Reuveni and Dirk Vuylsteke. Dr Novak contributed the *Musa* chapter for the earlier book, and it remains a fresh and challenging read even today. This book is dedicated to Oded Reuveni and Dirk R. Vuylsteke.

Oded Reuveni

Uri Lavi and Emanuel (Emi) Lahav

Institute of Horticulture, The ARO-Volcani Center, Bet-Dagan, Israel

Dr Oded Reuveni was a senior researcher at the Institute of Horticulture of the Volcani Center and its director for 8 years. He passed away in 1997 at the age of 64 after a serious disease. Oded was born in Rehovot, Israel, and from his childhood was interested in the secrets of plant growth. He used to tell the Chinese story of two penniless men who suddenly find two coins. One uses the money to buy food and the other buys food but also a flower explaining that the food is to sustain the body and the flower is to sustain the soul. Oded studied at the Faculty of Agriculture of the Hebrew University of Jerusalem, where he received all his academic degrees.

Dr Reuveni was a horticultural scientist and an agricultural expert – a very rare combination these days. He had tremendous knowledge, original ideas and the ability to think things through clearly. He encouraged research and development of unique areas and crops. He was a man of principles, always demanding high standards not only from himself but from others. He was always ready to learn and to continuously widen his scope of interest, and readily shared his experience, opinions and ideas.

An agricultural researcher has a choice of two avenues: (i) to focus on basic research while ignoring actual agricultural problems; and (ii) to solve practical problems without referring to the fundamental basis of the various phenomena. Dr Oded Reuveni preferred a combined approach, investing research efforts simultaneously in the current agricultural ‘bottlenecks’ and in the new horizons of basic research, which would bear fruit in the future.

Oded was a true horticulturist; he had a very broad knowledge of various fruit species, and had scientific curiosity to investigate those questions for which there were no easy

answers. He combined fundamental research on rooting, flowering and propagation of various species of fruit trees with the search for solutions to practical problems.

Dr Reuveni considered that his most important scientific contributions impacted plant propagation. For example, he pioneered the development of *in vitro* propagation of date palm, which has been commercialized in many countries. Banana also interested Oded, and his first *in vitro* plants were in the field in 1976. He noted the widespread occurrence of somaclonal variants, and proposed several causes of this problem and developed methods to identify variants at an early stage. He emphasized the importance of selecting stable and true-to-type sources for explants while minimizing the number of subculture cycles and the number of 'progeny' produced in culture. The banana industry worldwide owes much to this man. Dr Reuveni also developed methods and protocols for rooting of female papaya cuttings and for micropropagation of papaya which have been adopted in several countries. Oded developed procedures for rooting of cuttings of litchi cultivars and for avocado and mango rootstocks. He developed methods for rooting of cuttings of eucalyptus and for micropropagating this species. He also developed an efficient procedure for nursery production of forest trees based upon root air pruning, specific growth media and fertilization.

Dr Reuveni was deeply involved in various horticultural activities and was a 'guru' for many farmers. He investigated various aspects of the date palm: (i) assessment of leaf photosynthetic efficiency, which led to reducing the leafy area of a tree and increasing its yield; (ii) generation of a fertigation protocol based on tree requirements; and (iii) responses to salinity, which led to determination of the level of salinity which date palms can tolerate.

Dr Reuveni was the author of many scientific papers and review articles that dated from his first paper about flowering and pollination of loquat written with his teacher, the late Prof. C. Oppenheimer, to articles focusing on various aspects of date palm cultivation, i.e. fruit drop, bunch structure, pistil receptivity, trickle irrigation and propagation and rooting of date palm, avocado, eucalyptus, pine, papaya, banana and mango.

Dr Reuveni was very active in international committees and organized various international conferences. He was a major contributor to mango and banana research in developing countries. Dr Reuveni supervised several graduate students and participated in numerous scientific international symposia. He was a founder of the Biblical Fruit Society of Israel, whose goal has been the broadening of the knowledge of fruits grown in the Holy Land and strengthening the link between the people and the land. He was also a long-time member of the Israel Gene Bank, in which he invested great effort in preserving the local accessions of woody perennials, e.g. apple, figs and plum, *in situ* and *ex situ*, and he was one of the founders of the Israeli Society of Propagation.

Dr Reuveni's activities cannot be summarized solely on the basis of his formal scientific activities and achievements. Oded had an open door for farmers who appreciated his creative mind. Many farmers became his personal friends in spite of his demands for perfection and his uncompromising character. Gideon Ziv, a member of Kibbutz Yotvata in southern Israel, remembers Oded's suggestion to add activated charcoal to date palm pollen in order to 'label' the flowers which were pollinated. Dr Yair Israeli, a member of Kibbutz Ein Gev near the Sea of Galilee, was a friend and a colleague; Oded helped to establish the first banana micropropagation laboratories in Israel and Yair quotes Oded's advice, 'In order to be a good nursery person, you need to reject about 20% of your plantlets.' Visiting his friends on their farms was never a short formal visit but rather a long intensive workday, in which basic problems were addressed.

The horticulture farm at the Volcani Center was commemorated in Dr Oded Reuveni's name. Dr Oded Reuveni's death has been a great loss to his family, friends and the scientific community.

Dirk R. Vuylsteke

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Dirk R. Vuylsteke and his colleagues Paul R. Speijer and John B. Hartman tragically died on 30 January 2000, in the crash of a Kenya Airways aeroplane shortly after take-off from Abidjan, Côte d'Ivoire. The loss of these three young scientists was a great tragedy for their families, friends and colleagues, but also a significant blow to international agricultural research in *Musa*.

Dirk, a Belgian national, graduated from the Katholieke Universiteit Leuven (KUL) in 1981 with an MSc in Tropical Soil and Crop Science. He worked for some time at KUL on the development of a shoot-tip culture protocol for the propagation, conservation and distribution of plantain germplasm and, in 1982, he joined the International Institute of Tropical Agriculture (IITA) as an associate expert, where he worked with Rony Swennen, at the IITA High Rainfall Station in Onne (Nigeria).

Dirk's work on tissue culture contributed to the successful establishment of the *Musa* Germplasm Transit Center at KUL by the International Network for the Improvement of Bananas and Plantains (INIBAP). Dirk established an efficient micropropagation laboratory at the High Rainfall Station and produced training manuals for *Musa* micropropagation and field handling of *in vitro* plants.

To increase the output of hybrids from the *Musa* breeding programme, Dirk developed embryo culture techniques for improved *in vitro* germination of hybrid seed. This technique soon proved to be a key instrument in the breeding programme for plantain and banana, which was initiated at IITA in 1987.

The combined efforts of Dirk and Rony Swennen resulted in improved plantain and banana hybrids. The results of the research at IITA showed that segregation takes place in the triploid plantain genome during the modified megasporogenesis, leading to a new concept for improving bananas and plantains, whereby a vast array of new genetic combinations is generated through relatively few crossing operations.

In 1991, Dirk became leader of the IITA *Musa* research programme and in 1992 he was joined by a new breeder, Dr Rodomiro Ortiz. Together, they assembled knowledge about the genetics of black sigatoka and banana weevil resistance, and other important characters. In 1994, Dirk was appointed as Team Leader of the newly established IITA East and Southern Africa Regional Center (IITA-ESARC) in Uganda. As breeder, he took on the challenge of improving the East African highland bananas, a basic staple food crop in the Great Lakes Region. The first hybrids are now being tested in Uganda to assess their value and further use in *Musa* breeding for this country and others in the Great Lakes Region of Africa. Dirk managed a comprehensive programme for the genetic improvement of bananas and plantains and a new and very promising generation of hybrids, the secondary triploids, was created.

The impressive scientific productivity of Dirk Vuylsteke in less than 20 years can be judged from the number of his publications: 39 papers as senior author and 84 as a co-author. Besides his scientific achievements, I would also like to mention his caring personality. Dirk loved Africa profoundly and dedicated his life to working for the well-being of the smallholder farmers of this continent. He was a loved husband and father as well as a friend and respected colleague to many banana researchers.

Introduction

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Abstract

Major challenges lie ahead for farmers growing the world's fruit and nut crops, e.g. the difficulty of delivering increasing quantities of better quality, safer food while using fewer resources of land, water and inputs. Improvements, many made by scientific processes, in plant breeding and farming techniques have enabled world food production to keep pace with the increasing food requirements during the last millennium, but arguably at an ongoing and unsustainable cost to the environment. Biotechnology provides a set of powerful tools and approaches to improve plants or animals and to make or modify products. The application and exploitation of biotechnology in fruit and nut crops will undoubtedly make a major contribution to increasing the quantity, improving the quality and ensuring the sustainability of agriculture in this millennium, but biotechnology must be developed in conjunction with the requirements of farmers and end users, as well as moral imperatives regarding the environment and health, while issues of global access to appropriate technologies must be considered.

1. Introduction

Biotechnology, in the broadest sense, has been applied to the improvement of plants and plant products since their first selection for agriculture some 10,000 years ago. Developments have related not only to yield, quality and resistance characteristics of crops, but also to improving harvesting, storage and processing (including other biotechnological processes such as brewing and baking). In this chapter, I will discuss generic biotechnological approaches, new and old, that are available for use in fruit and nut crops, and consider some objectives for future crop improvement, with targets that may be important for individual crops, putting them into an environmental, social and economic context where responsible applications of biotechnology can make positive contributions.

The fruit and nut crop species discussed in this book represent 30% of the 150 primary food crops distinguished by the Food and Agriculture Organization (FAO), and together provide more than 15% of the world's human food supply. Many make critical contributions to the economies of the producer countries and a significant contribution to world trade:

virtually all are available in any Western supermarket. The major commodity crops, wheat, rice and maize, are critical to providing energy in diets. However, for the farmers that grow them, fruit and nut crops have much higher value and often make greater contributions to local economies and labour markets than other commodities.

2. Needs from Agriculture

Agricultural production increases are required across all crops during the 21st century; there are a billion people suffering from undernourishment in the world today, and the population is continuing to rise, albeit at a slowing rate compared to the mid-20th century. The biggest challenges may well be related to the global environment. Agricultural practices used over the last 1000 years have degraded the global environment and destroyed biodiversity; many current agricultural practices are considered to be unsustainable by many scientists. Water availability for agriculture may be diminishing and irrigation regimes are rarely sustainable in the long term, often exploiting non-renewable fresh water resources or degrading land through increasing salinity. Agricultural productivity per unit area, if not absolute production, must continue to rise. The balance and desirability of alternative strategies, ranging between large areas of lower intensity agriculture or smaller areas of highly intensive agriculture mitigated by 'preserved' habitats as refugia for native biodiversity and environments, are essentially social and political decisions. Progress in the crop sciences can increase the range of options available by providing crop varieties suitable for different agricultural systems and can also define the medium-term outcomes of different strategies.

Biotechnology must impact the quality of diets and safety, particularly of fruit and nuts. A diet based on cereals and/or root and tuber crops is deficient in amino acids, vitamins or provitamins and other micronutrients; fruit and nuts are part of any healthy diet, leading to a high quality of life. Consumers worldwide are demanding and receiving safer foodstuffs free of endogenous toxins or contamination from chemical and biological sources. Biotechnology in combination with health sciences can inform decision-making with respect to food safety, rather than offer absolute answers; many natural food components include antioxidants and anti-nutritional compounds that sometimes make foods unpalatable or toxic while improving health in another situation. Increases in lifelong allergies, particularly in people in developed countries, are being recorded, and may be caused by improved food hygiene.

Biotechnology is now making contributions to the development of fruit and nut crops ranging from the propagation of planting materials, through development of disease-free (particularly virus-free) stocks, to the directed breeding of new cultivars. These deliverables can increase production and sustainability of crops, and provide defined quality and safety characteristics. It is hardly conceivable that changes in agrichemical and other inputs to crops, in land area under cultivation or in agricultural technology can continue to deliver the food that will be required. Many agrichemicals are being withdrawn, often because of proven safety concerns; land is continuously lost to agriculture through urban development and leisure uses, and it is possible that rising sea levels will lead to loss of high-quality land. Horticultural practices will certainly continue to improve, e.g. minimal-tillage planting systems, systems involving underplanting or mixed cropping and introduction of rain-fed agriculture with trees for water management (increasingly practised in Australia). Many future changes may well be in conjunction with new cultivars with genetically determined characters making them suitable for particular cultivation conditions.

3. Cell and Tissue Culture

The vegetative propagation of plants by cuttings and grafting on to rootstocks has been practised for around 1000 years. Most fruit and nut crops have been routinely grafted for the last century. Perhaps the most widespread biotechnology introduced in the last three decades of the 20th century was plant cell and tissue culture. *In vitro* plant regeneration from existing meristems and somatic cells has been utilized for propagation and as part of the genetic manipulation strategy for many fruit and nut crops, many of which are of local importance.

Micropropagation is used routinely for a number of crops, e.g. for rapid multiplication of triploid banana selections and potato whose 'seed' tubers are widely produced from meristem cultures following thermotherapy to eliminate viruses. Nevertheless, there are many fruit and nut crops where improved *in vitro* regeneration methods from elite selections are still required for them to be routine and not dependent on genotype, with increased rates of conversion and reduced incidence of somaclonal variation. Embryo culture is essential for rescuing hybrid embryos, which would otherwise abort, e.g. papaya, or otherwise fail to develop in early fruit developing selections, e.g. peach. Improved regeneration of plants from single cells is critical for many perennial species from their mature phase (many of the crop species covered in this book) in order to fully exploit the induction of mutations or genetic transformation, both of which involve genetic manipulation of single cells.

4. Genome Analysis and Genomics

New methods of genome analysis, the science of genomics, in combination with the use of information from intensively investigated species, have the potential to enable rapid progress in the characterization of locally important and perennial species. Over the last 10 years, massive investments have been made in understanding plant genomes and the genes they carry in carefully chosen model species and a few crops of agricultural importance. Projects investigating genes, genetics, *in vitro* culture or genome organization that were carried out with tobacco, tomato, *Brassica* spp., *Arabidopsis thaliana*, rice and *Triticeae* cereals (wheat, barley) a decade ago are now achievable with realistic levels of investment in many other species; studies with perennial fruit and nut crops can exploit both the information and the data, and the technologies can then be applied. A number of worldwide initiatives have been remarkably successful with major crops such as rice. This volume demonstrates how biotechnology can now profoundly affect crop improvement and can be focused on the many challenges of crops, many of which are widely regarded as orphans where there is no major research base, e.g. mangosteen, carambola, etc. Common features affect their science and the application of technology, and international collaborations involving many researchers can be critical for exploiting new technologies when there are no major genomics programmes, large collections of germplasm or populations for genetic selection for such species.

Methods of analysis of plants at the DNA level have become notably more robust, simpler to use, not least because of the availability of trained people, and relatively less expensive. Genomic DNA isolation, cloning and sequencing have become routine, while methods such as the polymerase chain reaction (PCR) and methods for measuring DNA polymorphisms have developed such that polymorphisms can be identified and measured in any species. The genome sequencing projects have identified many plant genes and their functions, and homologous genes can be isolated and characterized in any other species with a straightforward, albeit time-consuming, programme.

Informatics and statistical analysis methods are allied to biotechnology. New mathematical methods and resources, including inexpensive computers and the Internet, are

leading to improvements in the accuracy and usability of genetic analysis and quantitative analysis of complex characters such as yield. Such tools will increasingly be applied to fruit and nut improvement programmes.

5. Plant Breeding

Many of the problems of breeding are unique to each species, as is clear from the coverage of the different crops in this volume: some are sterile and many require almost independent breeding processes for propagation and the harvested crop. Long-lived trees with juvenile periods greater than 5 years create special problems for traditional selection and evaluation, and grafted crop species require selection of different characters in rootstock and scion, which are complementary and interactive. In commodity crops, e.g. wheat and maize, the domesticated species is considerably different from the closest wild relatives, and for the most part they are grown well outside their native ranges. In contrast, many fruit and nut species are closer to their wild relatives, and the range of gene alleles is not unlike that in the wild. Hence, there is great potential for improving the yield, quality and stress resistance of these relatively unimproved selections, and perhaps to diversify the areas where they are grown, thereby reducing pressures from pathogens.

Despite the diversity in fruit and nut crops and differences in breeding methods, many of the objectives of plant breeders apply across a wide variety of species, and go far beyond characters related to yield and quality. Breeders are looking for selections with resistance to abiotic and biotic stresses, and robustness of yield to minimize season-to-season variation. Such challenges to plant breeders and crop production have common solutions offered by biotechnology.

Most breeding programmes aim to introduce genes that confer resistance to diseases. Involving continuous interplay between the pathogens and the crops and the environment, the process of identifying new disease resistances will never cease for any crop species. Molecular marker technology is helpful in assaying the presence and nature of disease resistance genes and ensuring transmission to cultivars, and can allow the pyramiding of multiple resistance genes in a cultivar.

Tree architecture, including shape and growth parameters, is very important. Self-pruning genotypes, which have the ideal shape for cultivation without requiring labour-intensive and skilled annual pruning, are valuable in many tree crop species: dwarf rootstocks for apples give better yields with less input of skilled labour for both pruning and harvesting. Small height increment may be important in many tree crop species. Growth characters are equally important for root systems, having major effects on accessing water (which may be seasonal and interactive with soil erosion) and plant stability. Control of plant shape and growth involves interaction of genetics and mathematical modelling in order to predict what selection should be used for optimum architecture.

Quality of products (and their attractiveness to consumers) can be as important as quantity, particularly for farmers aiming to sell into high-value markets. Genetic improvement of quality can be relatively simple, e.g. fruit colour, or involve manipulation of complex, often multigenic traits, such as seedlessness and parthenocarpy.

Estimates suggest that half of primary agricultural production is lost after harvest in many tropical regions after all the inputs, such as water, nitrogen and pest control, have been made to the crop. Transportation, storage, processing and marketing changes are needed to avoid these post-harvest losses, and improvements can be facilitated by genetic factors, e.g. easily predicted and controlled ripening, altered skin characteristics and better resistance to insects and pathogens that attack ripe fruits. Plant breeding objectives also respond to social pressures; in many developing countries, populations are moving from the countryside to cities, and new genetic crop characters may be more needed to make varieties suitable for

harvest, storage and transport, rather than use in subsistence and local agricultural systems. Worldwide, ease of harvesting and transportability have long been breeding objectives from the earliest domestication of crops when legumes and cereals were selected from wild types for non-shattering pods and non-breaking of rachides.

As perennial crop selection intensity increases and is better controlled during plant breeding, with widespread and rapid introduction of new and improved cultivars, the effects of new genes and gene combinations need to be considered for their impacts on consumers, production and the environment. With respect to food safety, it may well be possible to select a high-cyanide almond, which is highly resistant to insects, but also toxic to consumers. Less extreme, improved palatability through reduction of bitter compounds can make a plant more palatable to pests. Thus, application of biotechnology can enhance the safety of food. Suitable assays can also inform decisions about new cultivars; with some 0.5% of the population showing allergies to groundnuts (and 20% of these life-threatening), it is unlikely that groundnuts would be introduced as a new crop now; wheat, with perhaps a tenth of this frequency of allergic responses, would be borderline.

6. Diversity and Gene Sources

Genetic resources worldwide are the most important sources of new variation for plant breeders. Hence, there is a need for continuing collection and assessment of germplasm from all fruit and nut crops. The application of molecular marker methods enables efficient quantification of this diversity, although this must be in conjunction with phenotypic and physiological characterization. With annual crop species, after germplasm with valuable characters has been identified, the useful traits can be incorporated into elite cultivars by introgression, while undesirable characters will be removed by repeated back-crossing. In perennial fruit and nut crop species, this strategy is impractical due to the long juvenile period. Therefore, alternative strategies must be used.

7. Recombinant DNA, Gene Transfer and Genetic Modification

The range of molecular biology techniques available to plant breeders today includes not only the ability to measure and track genes and genetic variation, but also to directly move genes and their controlling elements between organisms, whether within or between species. Particularly in Europe, adoption of these methods has raised concerns, some of a general or philosophical nature relating to gene manipulation, the industrialization of agriculture and our food supply or dominance of technology by a small number of countries or companies. Given the ranges of genetic variation and natural flexibility of genomes, there is no scientific case to be made for ruling out all genetically modified (GM) crops and their products. There have been no verifiable ill effects reported from extensive growth and consumption of GM products in the last decade that are a consequence of GM technology. However, some groups of people have specific concerns related to either the impact on the environment of GM crops or the safety of food and animal feed derived from them. As discussed above, few would consider the agricultural methods currently providing the bulk of the world's food supply as being either environmentally benign or sustainable, e.g. farmers have become better at weed control every year for the ten millennia since the beginning of agriculture, reducing biodiversity in fields. Many plants have toxic or pharmacological properties, and breeders using conventional or GM technology transfer these to crops. With GM technology, defined genes can be transferred between genotypes, and the gene products can be analysed in detail. In contrast, novel germplasm, used either directly as a new crop or through sexual crossing, is harder to assess for safety because a range of novel proteins will be introduced and eaten by

human populations with no history of safe use. GM is not a single homogeneous technology and, as with conventional breeding, unsuitable genotypes can be produced. Thus, many would suggest that its applications should be considered and approved on a case-by-case basis, and regulation should be in line with new developments. The opportunity for correcting varietal weaknesses and improving fruit and nut crops through gene transfer are immense, and an excellent example of the use of such technology comes from virus resistance genes in papaya, which restored the Hawaiian industry. The developments in biodiversity characterization discussed above have much potential for identifying valuable genes for directed crop improvement both within and outside fruit and nut crops.

8. Prospects for Fruit and Nut Crops

The chapters of this volume discuss the range of biotechnological innovations that are now being applied to fruit and nut crops. Stakeholders at all levels, from the plant breeders, through farmers, processors and the supply chain, to the final consumers, whether in developed or developing countries, can benefit, and these crops have a critical role in enhancing sustainable agriculture, improving the environment and developing rural socio-economic systems. Without doubt, new developments in fruit and nut crops will play an increasing part in increasing the quality of life of the world's population.

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1

Actinidiaceae

The *Actinidiaceae* originated in temperate to subtropical regions of eastern Asia. This family includes only three genera, *Actinidia*, *Clematoclethra* and *Saurauia*, and is a member of the *Magnoliophyta* order of climbing woody vines. The genus *Actinidia* contains species with edible fruits. One of these, *Actinidia deliciosa* (A. Chev.) C.F. Liang and A.R. Ferguson, widely known as kiwifruit, has become a major crop worldwide. *Clematoclethra* fruits have no obvious economic use, while *Saurauia*, a large genus of about 300 species, has some with fruits that are eaten locally in Mexico and parts of Asia.

On the basis of morphological characters, > 60 *Actinidia* species have been distributed among four sections: *Leiocarpae*, *Maculatae*, *Strigosae* and *Stellatae* (Ferguson *et al.*, 1996). The taxonomy of the genus has had one

major revision over the last 30 years, with new species being added as they have been discovered. More recent molecular studies are clarifying species relationships.

While *A. deliciosa* and *A. chinensis* selections together are now the 'kiwifruit' of the international market, other species have potential as commercial cultivars, e.g. *A. arguta* with hairless, sweet, red- or green-fleshed fruit, the size of a large cherry, or could contribute desirable traits in breeding new cultivars. Flower and fruit variation found among *Actinidia* species illustrates the breeding potential of the available germplasm. Other potentially interesting species include: *A. kolomikta*, *A. callosa*, *A. eriantha*, *A. hemsleyana*, *A. latifolia*, *A. macrocarpa*, *A. melanandra*, *A. polygama* and *A. valvata*.

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1.1 *Actinidia* spp. Kiwifruit

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1. Introduction

1.1. Botany and history

Actinidia species are perennial climbing or scrambling vines. Most are deciduous, although a few which occur in subtropical areas are evergreen. All known species are functionally dioecious, indicating that dioecy preceded speciation (Ferguson, 1984), but a few individual hermaphrodite plants are known in *A. deliciosa*, a hexaploid species. The flowers are generally pentamerous with petals of white or various hues of cream, orange, yellow, pink and red. Male plants bear obviously unisexual flowers, with numerous stamens surrounding a rudimentary pistil, but female plants have flowers with hermaphroditic appearance. The pistillate flowers have a gynoeceum of numerous carpels fused at the ovarian level and free styles radiating in a whorl, all surrounded by fully developed stamens. However, microspores in pistillate flowers degenerate after tetrad release, before microspore mitosis (Harvey *et al.*, 1997). Recent biochemical studies indicate that male sterility in *A. deliciosa* may be under both sporophytic and gametophytic control (Biasi *et al.*, 2001). A pollen tube distributor cup, located on the columella at the distal end of the ovary, ensures that pollen tubes

travelling through the transmitting tract are evenly distributed around the ovary, resulting in an even distribution of seeds in the fruit (Howpage *et al.*, 1998). Abundant secretion present along the entire pollen tube pathway, from stigma to ovules, appears to be involved in pollen nutrition and guidance, disappearing after pollen tube passage, a feature that could explain the high reproductive success of *Actinidia* (González *et al.*, 1996). The fruits, oblong or spherical berries, have a large number of small seeds in a central circle of locules in a fleshy pulp.

Palaeobiology studies revealed that *Actinidia* is at least 20–26 million years old (Qian and Yu, 1991), and there are records of its use dating from the Tang dynasty in China (618–907) (Lee, 1990). China, the centre of diversity of the genus, has the most abundant and diverse *Actinidia* resources of wild germplasm. Over 60 species have been identified, and numerous selections have been made, including genotypes with cold or drought tolerance or wide climatic adaptability (Qian and Yu, 1991).

Actinidia species were brought from China to Europe during the second half of the 19th century and early 20th century. Only at the beginning of the 20th century was *Actinidia* taken to New Zealand, where selection revealed commercially interesting culti-

vars. The first commercial plantings of kiwifruit were made in New Zealand in the early 1930s, and currently total world production of fruit is reported to be 995,517 Mt (FAOSTAT, 2004).

A. chinensis and *A. deliciosa* were, until recently, considered to be a single species, as morphologically they are very closely related. However, restriction fragment length polymorphism (RFLP) analyses with both nuclear and chloroplast probes suggested a reasonable level of distinctness between their genomes, and these data, together with some morphological detail, supported their classification as distinct species (Crowhurst *et al.*, 1990; Ferguson *et al.*, 1996).

Isoenzyme polymorphism analyses suggested *A. chinensis* might be a precursor of *A. deliciosa* (Huang *et al.*, 1997; Testolin and Ferguson, 1997), a hypothesis supported by RFLP analysis (Crowhurst *et al.*, 1990), flavonoid composition and *in situ* hybridization with repeat DNA and genomic probes (Yan *et al.*, 1997). Yan *et al.* (1997) verified that both *A. deliciosa* and tetraploid *A. chinensis* have six hybridization sites with the repeat sequence pKIWI516. This points to the most likely origin of hexaploid *A. deliciosa* being via tetraploid forms of *A. chinensis*, without contributions from other *Actinidia* species, and possibly involving the formation of unreduced gametes (in Testolin *et al.*, 1999). Such an origin of *A. deliciosa* was also suggested by Atkinson *et al.* (1997), based on phylogenetic analyses of DNA sequences derived from the polygalacturonase (PG) gene.

Restriction analyses of PCR-amplified sequences of chloroplast DNA, however, revealed some discrepancies between the morphological and biochemical traits, and suggested a widely reticulate evolution within the *Actinidia* genus (Cipriani *et al.*, 1998). With these data, the division of the genus into four groups of species appears to be weakly supported.

Microsatellites have been used in *Actinidia* fingerprinting (Weising *et al.*, 1996), and were developed and tested in progenies of controlled crosses to provide markers for genotype selection in breeding programmes

(Huang *et al.*, 1998). Data from this work supported a previous suggestion by McNeilage and Considine (in Huang *et al.*, 1998) that *Actinidia* ancestors may have had a chromosome number $x = 15$ and/or 14. This hypothesis pointed to a basic chromosome number similar to *Saurauia* ($2n = 30$), and assumed that the genus could have evolved to a stable tetraploid level ($2n = 58$) (Huang *et al.*, 1998).

Unlike most angiosperms, *Actinidia* has a strict paternal plastid inheritance, demonstrated in interspecific crosses by PCR amplification of chloroplast DNA (Cipriani *et al.*, 1995). Testolin and Cipriani (1997) confirmed this situation in seedlings of interspecific crosses, and verified that, at the mitochondrial level, *Actinidia* followed the general rule in plants of maternal inheritance. Identical results were obtained for intraspecific crosses (Chat *et al.*, 1999). Apparently, the modes of inheritance of mitochondria and plastids (paternal/biparental or maternal) are determined independently of each other in young generative cells just after pollen mitosis I (Nagata *et al.*, 1999).

1.2. Importance

Actinidia fruits are highly nutritious. All species are rich in Ca, K, Fe, Mg, mineral nutrients, amino acids and dietary fibre. They have a particularly high vitamin C content, with values > 30 mg/100 g fresh weight of fruit (80–300 mg in *A. deliciosa*, and reaching 1000 mg in *A. kolomikta*).

One of the more important properties of *Actinidia* fruits is the anti-mutagenic activity of the juice, first recorded in popular medicine and later investigated in scientific studies. Cancer inhibition in the digestive system and effective blockage of the synthesis of carcinogenic nitrite compounds have been reported (Lee and Lin, 1988). From 36 anticancer agents used in Chinese medicine, *A. chinensis* was one of the three that completely inhibited the mutagenicity of benzo[a]pyrene and among six with moderate protection against picrolonic acid-induced mutation (Lee and Lin, 1988). Other medicinal properties attributed to

Actinidia fruits include relief of cardiovascular diseases and strong laxative effects (Qian and Yu, 1991). The juice, prepared in water or syrups, is said to diminish inflammation and cure shortness of breath and coughing (Gao and Xie, 1990).

Adverse effects of kiwifruit have also been recorded; fruits can cause dermatitis (urticaria), swelling and itching of lips and tongue and stomach pains, thought to be due to the needle-shaped calcium oxalate crystals (raphides) and the proteolytic enzyme actinidin (Zina and Bundino, 1983; Perera and Hallett, 1991). Actinidin is a plant thiol proteinase, as are papain (papaya), ficin (fig) and bromelain (pineapple).

Actinidia fruits attractive to the consumer, with increased nutritional value and improved characteristics for producers and marketers, may also be achieved through biotechnology. Ploidy manipulation, tissue culture and gene transfer techniques, together with marker technology, may significantly accelerate the breeding process (Ferguson *et al.*, 1996).

1.3. Breeding and genetics

Due to the comparatively recent domestication of kiwifruit only one cultivar, 'ZespriTM Gold', has so far emerged from systematic breeding programmes and been a commercial success on the world markets. *A. deliciosa* and *A. chinensis* are the only widely cultivated *Actinidia* species, but other species, such as *A. arguta*, are possible candidates for commercial development. Work is focusing on selection of improved genotypes from intraspecific breeding programmes and the production of new cultivars from interspecific crosses. Extension of the harvest period, improvement of vine tolerance to adverse conditions, disease resistance, improved yield and fruit characters, e.g. flavour, sweetness, texture and flesh colour, are all goals being targeted.

The genus has a reticulate polyploid structure, with diploids, tetraploids, hexaploids and octaploids occurring in diminishing frequency (Ferguson *et al.*, 1997). The basic chromosome number in *Actinidia* is

high at $x = 29$, with a diploid number of 58. Polyploids seem to contain three, four, five, six or eight times the basic 29 chromosomes (Yan *et al.*, 1994). Chromosome analysis and flow cytometry have shown that several *Actinidia* species contain ploidy races. *A. chinensis* has diploid and tetraploid races while *A. arguta* is generally tetraploid, but both diploid and hexaploid specimens have been reported and *A. arguta purpurea* has been shown to be octaploid. Diploid and tetraploid forms of *A. callosa*, *A. kolomikta* and *A. polygama* and tetraploid and hexaploid forms of *A. valvata* have been recorded (Ferguson *et al.*, 1997).

Hybrids have been produced between tetraploid *A. chinensis* and *A. deliciosa* and between diploid and tetraploid races of *A. chinensis* (Ferguson *et al.*, 1997). The introgression of characteristics of interest from one species into another requires knowledge of the relationships between taxa.

1.3.1. Rootstocks

Major breeding objectives. For several years kiwifruit vines in commercial orchards were not grafted, but the need to expand the culture to less appropriate soils and the low resistance of *A. deliciosa* to cold winters obliged the industry to look for appropriate rootstocks. Rootstocks are of value in the development of sustainable production methods, with minimal use of insecticides and fungicides. In Italy a clonal rootstock ('D1') is often used due to its tolerance of calcareous soils, while in New Zealand the rootstock 'Kaimai' ('TR-2') enhanced flowering of 'Hayward', especially in younger vines (in Ferguson *et al.*, 1996).

Breeding accomplishments. There are no reported applications of the use of biotechnology for rootstock breeding for *Actinidia*, but populations from breeding programmes are screened for potential rootstocks (Ferguson *et al.*, 1996). *A. deliciosa* 'Hayward' grafted to seedling rootstocks of other *Actinidia* species reduced the level of floral abortion and increased bud burst (Wang *et al.*, 1994a). This occurred with *A. hemsllyana*, *A. eriantha* and *A. rufa* rootstocks (110%, 73%

and 30% increase in flower production, respectively), while *A. chinensis* rootstock had the opposite effect (23% reduction).

The roots of flower-promoting rootstocks tend to have more and larger xylem vessels, more starch grains and more and larger idioblasts with raphides and mucilage (Wang *et al.*, 1994b). Mucilage holds water, and these anatomical differences may thus be linked to improved water relationships and have been suggested as selection criteria for better rootstocks.

A. deliciosa is a host for vesicular-arbuscular mycorrhizal fungi. Powell and Santhanakrishnan (1986) found that mycorrhizal inoculation increased shoot length and weight by 294 and 265%, respectively, in hardwood cuttings, and suggested rootstock mycorrhization should also be explored for optimal fruit production in the scion.

1.3.2. Scions

Major breeding objectives. Diseases and infestations may constitute a problem in *Actinidia* orchards (in Jordán and Botti, 1992). Caterpillars and scale, such as greedy scale (*Hemiberlesia rapax*), can affect unsprayed kiwifruit vines (Blank *et al.*, 1996). The root knot nematode, *Meloidogyne hapla*, among other species, causes severe root deformations, and *M. hapla* has been associated with poor fruit size and uneven ripening and with vine decline. The kiwifruit bacterial blossom blight, caused mainly by *Pseudomonas viridiflava*, can lead to loss of buds and flowers, and occurs more frequently in warm, moist environments. Fungi like *Armillaria mellea* (oak root fungus) and *Phytophthora* spp. appear frequently in wet soils, and *Botrytis cinerea* causes significant postharvest losses in kiwifruit (Michailides and Elmer, 2000). *Botryosphaeria* spp. and *Phomopsis* spp. can also cause severe problems of rot in ripe fruit (Nakamura *et al.*, 1999).

Characteristics of the fruit to improve or alter include size, flavour, colour, core softness, nutritional quality (control of the content of actinidin, calcium oxalate or the constituents responsible for the laxative effect) and hair (preferably the loss of fruit hair at maturity to reduce skin blemish or

damage). A tendency to produce flat or fan-shaped fruits should also be avoided. Variations in harvest dates could be important, to reduce the concentration of seasonal work at harvest time and to expand market opportunities. For selection of these characteristics in breeding programmes the use of DNA fingerprinting and marker-assisted selection could be extremely helpful.

Breeding accomplishments. Several breeding programmes of *Actinidia* were initiated in the past two decades, mainly in New Zealand and Italy, where the industry has been dependent on 'Hayward' (Harvey *et al.*, 1998). Germplasm from China was imported into various countries, and research on molecular markers and genetic pathways linked to commercially important features in the species has been initiated.

Breeding programmes were initially based on seedling selections from progenies of open-pollinated or crossed *A. deliciosa* genotypes, followed by intra- and interspecific crosses among other species. Characterization of breeding parents has been, and is, an important feature of breeding programmes. Multivariate analysis of variance (MANOVA) revealed that *A. deliciosa* 'Bruno' was a better female parent for production of floriferous male seedlings, with a long bloom period, and that D-1-20 was a male parent which produced more floriferous progeny with stronger early vigour (Daoyu and Lawes, 2000).

The occasional occurrence of fruiting males in hexaploid *A. deliciosa* was exploited by Hirsch (in Ollitrault-Sammarcelli *et al.*, 1994), who succeeded in producing 40 individuals from *in vitro*-cultured seeds removed from one such male ('Lea'). Flow cytometry of these plants revealed seven with a deduced ploidy level of 9x. Five of these remained juvenile 4 years after planting, and a sixth one remained dwarf (Ollitrault-Sammarcelli *et al.*, 1994).

In interspecific crosses, the percentage of hybrid seed is usually reduced, possibly because of the difference in ploidy level and physical parameters of the flowers of the species crossed, and both embryo and endosperm failure has been observed

(Harvey *et al.*, 1991, 1995). Embryo rescue was attempted to overcome the problems, and also to provide material for genetic studies (Mu *et al.* in Harvey *et al.*, 1991; Cipriani *et al.*, 1995). In the cross *A. chinensis* var. *chinensis* (2x) $\times$ *A. melanandra* Franch. var. *melanandra* (4x), embryo rescue and colchicine treatments produced plants of 2x, 3x, 4x and 6x (Harvey *et al.*, 1995). In crosses where 'Hayward' was pollinated by *A. arguta* (4x) and *A. eriantha* (2x), viable hybrids were produced, 50% of which were tetraploid (Mu *et al.*, 1991).

To support breeding programmes, random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) marker techniques are being used (Cipriani *et al.*, 1996; Harvey *et al.*, 1998; Fraser *et al.*, 2001), and a linkage map has been constructed with SSRs and AFLP markers (Testolin *et al.*, 2001).

2. Molecular Genetics

2.1. Gene cloning

Interest in *Actinidia* genes has focused mainly on those expressed in the fruit. Several genes have been cloned from fruit complementary DNAs (cDNAs), such as actinidin (Podivinsky *et al.*, 1989), a homologue of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (MacDiarmid and Gardner, 1993), ACC synthase and ACC oxidase genes (Whittaker *et al.*, 1997), three sequences encoding S-adenosyl-L-methionine (SAM) synthase (Whittaker *et al.*, 1995), three PG genes (Wang *et al.*, 2000), a PG-inhibiting protein (Simpson *et al.*, 1995) and six cDNA clones homologous with xyloglucan endotransglycosylase (XET) (Schröder *et al.*, 1998). Five cDNAs for differentially expressed genes have been cloned from a library made from young fruits, two with homology to plant metallothionein-like proteins (Ledger and Gardner, 1994). cDNA sequences of sucrose-phosphate synthase (SPS) have also been cloned from *Actinidia* (Langenkämper *et al.*, 1998). An *Actinidia* genomic library has

been used to clone a PG gene by probing with a tomato PG cDNA clone (Atkinson and Gardner, 1993). Recently, interest in the development of molecular probes to investigate floral commitment led to the cloning of *LEAFY* and *APETALA1* homologues, named respectively *ALF* and *AAP1*, from *Actinidia* (Walton *et al.*, 2001).

The identification of genes involved in sex determination has been a research goal, to manipulate pollen fertility in the female or ovule and ovary development in the male. cDNA clones have been obtained from male and female flower buds at pre- and post-meiotic stages of microspore development and differentially screened (Fraser *et al.*, 1997). Clones from a male post-meiotic library have shown homology with stamen- and/or tapetum-specific proteins of other plants.

2.2. Marker-assisted selection

The development of molecular strategies for early sex identification has been a priority in breeding programmes and, in the first studies, peroxidase markers were used (Hirsch *et al.*, 1991).

A monofactorial system, based on an active Y chromosome, similar to the system described for *Melandrium album* (Ye *et al.*, 1991), was proposed that could justify the conservation of the disomic sex control in polyploids. Any plant with a single Y chromosome, even when several X chromosomes were present, would be a male (Testolin *et al.*, 1999). A 1:1 male:female progeny segregation was observed in *Actinidia* diploids and hexaploids, although a deviation towards an excess of males was previously reported, apparently because males are more precocious in overcoming juvenility (Testolin *et al.*, 1999). This model assumes that sex chromosomes undergo crossing-over, except in the differential segment(s) where the sex-determining genes are located. At least two genes must be involved in sex expression, one to suppress pistil development in male phenotypes and the other to cause pollen death in females (Harvey *et al.*, 1998).

Two markers linked to the Y and the X chromosomes (SmY and SmX) were identified using RAPD markers in a bulk segregant analysis of DNA pooled from male and female siblings of *A. chinensis* (Harvey *et al.*, 1997). Both markers were inherited from the male parent. The SmY marker could be used to identify male plants among the progenies, while SmX could distinguish gender depending on the inheritance of the X chromosome. The segregation of the SmX marker (850 bp) together with the female phenotype is shown in Fig. 1.1.1.

These markers were converted into sequence-characterized amplified regions (SCARs). The conversion was successful with SmX, but SmY failed to retain polymorphism (Gill *et al.*, 1998). To regain polymorphism between the genders, the alternate allele from females was cloned and sequenced and primers were designed that were specific for this allele. The SCAR markers were applicable in *A. chinensis* accessions from different geographic regions and the closely related *A. deliciosa* (but not in other *Actinidia* species), and can now be used for large-scale screening of seedling populations in *Actinidia* breeding. AFLP polymorphic bands have also been amplified from male

and female DNA bulks, and a male-specific band (AF5) was closely linked to the sex locus and present only in the male plants (Zhang *et al.*, 2000).

The suggestion that, in *Actinidia*, dioecy preceded speciation supports the feasibility of isolating a genus-wide sex marker (Harvey *et al.*, 1998).

2.3. Functional genomics

Several studies designed to gain an understanding of the regulation, mainly at fruit level, of genes identified in *A. deliciosa* or in *A. chinensis* have been undertaken. Whittaker *et al.* (1997) correlated the expression of ethylene biosynthetic genes in developing, ripening and senescing fruit of *A. chinensis* with ethylene levels in the fruit and proposed that the induction of SAM synthase transcripts by ethylene may occur as part of the methionine salvage pathway.

Although *Actinidia* fruit ripening is climacteric, part of the softening process is independent of ethylene production. Wang *et al.* (2000) studied, in several tissues, the expression of three PG cDNA clones obtained from fruits. The three PG genes

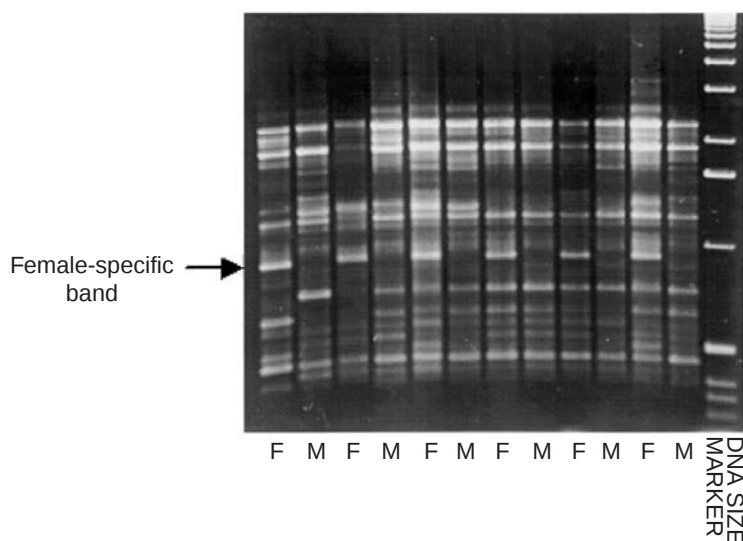


Fig. 1.1.1. RAPD fingerprinting of kiwifruit, illustrating the segregation of the 850 bp SmX marker, together with the female phenotype.

were expressed in stage III of fruit ripening, but one was also expressed in stages I and II.¹ The authors concluded that PG expression is required not only during periods of cell wall degeneration, but also during periods of cell wall turnover and expansion. Promoter fragments of one PG clone driving the β -glucuronidase (*uidA*) gene were tested in transgenic tomato (Wang *et al.*, 2000). This strategy revealed PG gene expression in softening fruit tissue prior to ethylene production associated with the respiratory climacteric.

Langenkämper *et al.* (1998) observed the increase of SPS in response to a substrate sourced from starch degradation, as well as to ethylene and low temperature, during fruit ripening. The authors suggested that SPS might play a central role in regulating carbohydrate fluxes at that time.

Six cDNA clones with homology to XET, cloned from ripe *A. deliciosa*, apparently belong to a family of closely related genes (Schröder *et al.*, 1998). These genes were induced in ripening fruit when endogenous ethylene production was first detected, and peaked in climacteric samples when fruits were soft. A full-length XET cDNA expressed in *Escherichia coli* showed endotransglycosylase activity when refolded. The authors postulated its activity in fruit ripening in rapidly expanding cells as cutting and rejoining xyloglucan intermolecular tethers between adjacent cellulose fibrils, thus restoring the original strength of the cell wall, and during senescence by irreversibly breaking xyloglucan tethers between cellulose fibrils. Based on the three to five hybridization copies detected in Southern analysis in *A. chinensis*, the authors estimated that up to 30 copies of the XET gene family may be present in *A. deliciosa*.

The promoter of the most abundant actinidin gene from the multigene family was studied in transgenic petunia plants dri-

ving the *uidA* coding region (Lin *et al.*, 1993). The pattern of late fruit expression observed in *Actinidia* was maintained in petunia, supporting the hypothesis that the genetic mechanisms controlling development and tissue specificity are evolutionarily conserved in many *Dicotyledoneae*.

The mechanisms of actinidin processing and accumulation were investigated in another heterologous system, tobacco, using the cauliflower mosaic virus (CaMV) 35S promoter and a tapetum-specific promoter (Paul *et al.*, 1995). In tobacco, the correct processing of the protein from preproactinidin required the presence of the C-terminal propeptide, which, in actinidin, has 34 amino acids, one of the longest described. The difference in actinidin accumulation between *Actinidia* species (~60%) and tobacco (8%) suggested *Actinidia* may have additional mechanisms to allow such high accumulation (Paul *et al.*, 1995). Preliminary results indicated a vacuolar accumulation, and *in vitro* studies pointed to the involvement of other proteases, probably vacuolar, necessary to convert preproactinidin into mature actinidin.

A thaumatin-like protein, purified from woody tissue of *Actinidia*, has shown a high degree of homology with basic endochitinase in orange and thaumatin-like proteins of tobacco, maize, barley and tomato (Wurms *et al.*, 1999).

The cDNA clones *ALF* and *AAP1* of *Actinidia*, homologues of the *Arabidopsis* floral meristem identity genes *LEAFY* and *APETALA1*, respectively, were used in *in situ* hybridization in *A. deliciosa* to understand the regulation of flowering (Walton *et al.*, 2001). The studies revealed that floral commitment (evocation) was likely to have occurred early in the first growing season of bud development when second-order meristems were initiated, and again 10 months later when those meristems differentiated flowers.

¹ Stage I – Fruits do not soften immediately after harvest unless exogenous ethylene is applied; stage II – starch degradation occurs, pectin methylesterase activity increases and soluble pectin is degraded, xyloglucan molecular weight decreases and cell walls swell; stage III – characterized by respiratory climacteric, production of endogenous ethylene and synthesis of aromas and volatiles (MacRae and Redgwell, in Wang *et al.*, 2000).

3. Micropropagation

The expansion of the kiwifruit industry led to an increasing demand for propagating material. The conventional propagation methods, by cuttings and grafts, were supplemented by *in vitro* micropropagation in some countries. Micropropagation was first described by Harada (1975), but numerous studies were published in the following years (see Ferguson *et al.*, 1996, for review). The presence of contaminating bacteria inside kiwifruit tissues was reported but this did not constitute an obstacle to efficient use of the method (Monette, 1986).

In general, actively growing shoots were surface sterilized using sodium hypochloride (0.6–1.5%) for 15–30 min, in the presence of a detergent (1% Tween 20), and single nodes have been cultured on semi-solid MS basal medium (Murashige and Skoog, 1962) with 20 g/l sucrose, supplemented with 4.6 μM zeatin and 0.3 μM indole-3-acetic acid (IAA), or 4.6 μM zeatin, 2.9 μM gibberellic acid (GA_3) and 2.2 μM benzyladenine (BA). Often, the MS medium strength has been reduced to 75% or 50%. Kiwifruit shoot cultures have been maintained under a photoperiod of 16 h with variable light intensities (usually 25–35 $\mu\text{mol photons/m}^2/\text{s}$) and temperatures of $22 \pm 2^\circ\text{C}$.

A high shoot proliferation rate was reported using liquid medium with 8.9 μM BA and 0.3 μM indolebutyric acid (IBA), and chilled lateral buds were the best for multiplication (Lionakis and Zirari, 1991). Marino and Bertazza (1990) verified that BA caused hyperhydricity of older leaves, an effect not observed with zeatin, but higher proliferation rates.

Efficient proliferation has been achieved using a 4-week subculture period on 50% MS macro- and micronutrients, full-strength MS vitamins, 5 mg/l ascorbic acid as antioxidant and supplementation of 2.3 μM zeatin and 0.3 μM IAA (Pais *et al.*, 1987). Nodes taken from these shoots and transferred to identical medium, with 5 mg/l dithiothreitol (DTT) replacing ascorbic acid and devoid of growth regulators (medium H2), produced shoots with no basal callus in 5 weeks, 1.8

roots per shoot, 93% survival success and a normal appearance (Pedroso *et al.*, 1992). This medium could also be used for normal shoot proliferation, with the advantage of eliminating aberrant forms often described for media containing growth regulators (Rodríguez *et al.*, 1985).

In most cases, rooting has been achieved upon dipping the shoot bases in IBA and transferring to well aerated soil mixtures. Many different auxin concentrations have been used with success for variable periods of time (e.g. 2.5 μM IBA in the culture medium for 1 month, or dipping in 4.9 mM IBA for 20 s). Monette (1986) reported no difference in the number of roots obtained with 2.5, 4.9 or 9.8 μM IBA, and recommended dipping in 2.5 μM IBA and direct rooting in a peat:vermiculite:perlite mix. Acclimatization has been achieved by gradually reducing the humidity from 100% in hardening chambers (Marino and Bertazza, 1990) or in nurseries with 80% relative humidity for 30 days with 16 h photoperiod and reduced light (8.6 $\mu\text{mol/m}^2/\text{s}$) (Pedroso *et al.*, 1992). Shen *et al.* (1990) also referred to the importance of reducing light during hardening of *A. deliciosa* and *A. chinensis*.

The performance in the field of micro-propagated plants was compared to that of cuttings and grafts, and found to be the poorest (cuttings being the best). There were a 1-year delay in flowering and lower efficiencies in energy uptake and partitioning of photoassimilates, due to tissue culture-induced juvenility (Díaz Hernández *et al.*, 1997). Piccotino *et al.* (1997) found that, in comparison with plants obtained from woody cuttings, micropropagated plants had xylem vessels of reduced size and a lower quantity of epicuticular waxes, among other anatomical and morphological modifications, but did show an increased tolerance of frost damage.

Aseptic encapsulation of *Actinidia* micro-cuttings in sodium-alginate beads has been used to evaluate aptitude for synthetic seed production (Adriani *et al.*, 2000). Cold treatment of the mother shoots and increase of sucrose concentration in some steps of the protocol enhanced the conversion to whole plantlets.

4. Micrografting to Eliminate Viruses

Virus diseases have not been described in kiwifruit orchards or in other *Actinidia* species. Although kiwifruit showed susceptibility to some viruses common in Italy, no virus could be isolated from field grown *Actinidia* plants (Caciagli and Lovisolo, 1987). However, a new disease transmitted by *A. polygama* rootstock to eight varieties of kiwifruit was observed in Japan, with symptoms developing 2 years after grafting and only visible on the scion (Nitta and Ogasawara, 1997). The disease agent, causing chlorotic lines, spots, rings and yellow mosaic patterns, was not identified.

Micrografting (*ex vitro*) was reported to accelerate only by 2–3 years the growth and fruiting of *Actinidia* protoplast-derived clones (Ke *et al.*, 1993).

5. Somatic Cell Genetics

5.1. Regeneration

5.1.1. Somatic embryogenesis

Several papers dealing with tissue culture in *Actinidia* have reported induction of embryogenic cultures; however, in only a few cases has this process been specifically addressed (see Oliveira, 1999, for review). When male and female genotypes of *A. chinensis* and *A. deliciosa* were tested for their embryogenic potential, *A. chinensis* was the more responsive species, although the embryogenic response was highly genotype-dependent. Stems and roots, leaves, anthers and filaments have all been used as explants, but consistent embryo conversion into plantlets was only demonstrated from the anther wall callus (Fraser and Harvey, 1986).

Anthers from preconditioned flower buds of *A. chinensis* genotypes and *A. deliciosa* 'Matua' were used as explants for induction of embryogenic cultures, from which plantlets developed. The hormone 2-isopentenyladenine (2iP) at 25–50 μM was used for induction on LS (Linsmaier and Skoog, 1965) or B5 (Gamborg *et al.*, 1968) basal media. Embryo germination was achieved with

1.7 μM IAA and 25 μM 2iP in *A. deliciosa*, while in *A. chinensis* the transfer from darkness to photoperiod conditions was enough to promote differentiation (Fraser and Harvey, 1986). For both species the ploidy level of the regenerated plants was the same as that of the mother plants, indicating their somatic origin.

Direct embryogenesis has only been reported from *in vitro* leaves of 'Hayward', although the embryos did not germinate well (Oliveira and Pais, 1992). In this case, explant preconditioning (cold and starvation) was essential for induction and it was achieved by maintaining shoots, devoid of leaves, in the cold (4°C) and dark for 45 days in Petri dishes sealed with Parafilm®. After this period, the axillary nodes were excised and cultured on 50% MS medium with 9.1 μM zeatin and 40 g/l glucose. The first to third leaves taken from the growing shoots showed embryogenic potential after 1–2 months of culture in the dark, on 50% MS medium with full-strength MS vitamins, 20 g/l glucose or sucrose and 9.1 μM zeatin. Somatic embryos always developed on the leaf veins on entire or half leaves, on the adaxial surface facing the culture medium. Embryo induction only occurred within the culture medium, probably indicating a requirement for anaerobic stress, similar to the *in ovulo* environment (Oliveira, 1999). Somatic embryo germination was achieved only occasionally on 50% MS medium with full-strength vitamins, no growth regulators and under a 16 h photoperiod with reduced light (10–12 $\mu\text{mol}/\text{m}^2/\text{s}$).

Although *Actinidia* tissues have embryogenic competence, the system is still insufficiently defined to be useful for clonal propagation or genetic manipulation and must be extended to more genotypes and explants.

5.1.2. Organogenesis

Many different strategies have been applied to a variety of explant types of *Actinidia* species to achieve plant regeneration through *de novo* shoot induction. Stem, leaf blade, petiole, root, filament, anther, cotyledon, and callus and cell suspensions from

various explants have all demonstrated organogenic potential. In comparison with leaf blades, petioles or stems, roots have the lowest regenerative ability. Most studies involved *A. deliciosa* 'Hayward', but other female ('Monty', 'Bruno', 'Andreana') and male cultivars ('Matua', 'Tomuri') were also used, as well as other species (*A. chinensis*, *A. arguta*, *A. eriantha*, *A. polygama* and *A. kolomikta*) (see Ferguson *et al.*, 1996, for review; see also Gui, 1979; Canhoto and Cruz, 1987; Nayak and Beyl, 1987; Pais *et al.*, 1987; Chiariotti *et al.*, 1991; González *et al.*, 1995; Famiani *et al.*, 1997; Tanaka *et al.*, 1997).

Several cytokinins were effective in inducing organogenic cultures from *Actinidia* tissues. Thidiazuron was reported to be the most effective for shoot induction from cell suspensions (Suezawa *et al.*, 1988) and from callus of *A. deliciosa*, *A. polygama* and *A. kolomikta* (Nayak and Beyl, 1987). Another phenylurea (*N*-2-chloro-4-pyridil-*N*-phenylurea (4PU)) was also indicated for shoot induction from cell suspensions (Suezawa *et al.*, 1988) and from calluses in transformation experiments (Matsuta *et al.*, 1990, 1993; Uematsu *et al.*, 1991; see Section 5.2.3). In most cases, however, shoot induction was achieved with zeatin or BA, and the use of 2iP was reported only occasionally. To achieve regeneration from suspension cultures, Suezawa *et al.* (1988) established leaf-derived callus on solid basal medium with 5 μ M naphthaleneacetic acid (NAA) and 1 μ M zeatin in darkness at 28°C for 40 days. Induced calluses, transferred to medium with 5 μ M 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 μ M zeatin, appeared soft and friable and appropriate for suspension cultures, whereas those on NAA were nodular and firm. The addition of 5 mM glutamine increased growth of the cell suspensions, which were maintained in darkness with shaking (100 rpm). Suspensions, spread as a thin layer on to medium containing 50 μ M NAA, 10 μ M zeatin, 5 mM glutamine and 0.1% casein hydrolysate, formed colonies with a nodular appearance. Such colonies, when transferred on to semi-solid medium with 50 μ M thidiazuron and under a 16 h photoperiod of 35–40 μ mol/m²/s, developed adventitious buds within 1 month. The

beneficial effect of NAA for inducing organogenic competence prior to the cytokinin signal was also observed for protoplast-derived cultures (see Section 5.15). Oliveira (1992) observed loss of organogenic competence in calluses maintained on media with 4.5 μ M 2,4-D and 0.5 μ M kinetin. Recovery of competency was achieved if an intermediate step of culture on 5.4 μ M NAA and 0.5 μ M zeatin was introduced prior to transfer to MS medium with 4.6 or 9.1 μ M zeatin and 0.1 μ M IAA.

Stem segments of female clones of *A. deliciosa* showed higher organogenic potential than those of male clones (Gui, 1979). In contrast, stamens from male clones produced callus and shoots more readily, and those from female clones were more rhizogenic (Brossard-Chriqui and Tripathi, in Ferguson *et al.*, 1996). In the presence of 2iP, roots of the male 'Tomuri' regenerated shoots, in comparison with those of 'Hayward', which did not (Chiariotti *et al.*, 1991). Hirsch and Bligny-Fortune (1979) also reported that stem-derived cell suspensions of female genotypes of *A. deliciosa*, cultured in the presence of 2,4-D, tended to lose chlorophyll, in contrast to male genotypes. These different behaviours may be related to variations in endogenous cytokinin and auxin levels in male and female tissues. Marino and Bertazza (1990) also suggested a higher cytokinin:auxin endogenous balance for 'Tomuri' than for 'Hayward', based on chlorophyll content and proliferation rates of micropropagated shoots of both cultivars.

High levels of sugars in the culture medium of 'Hayward' increased lignification and reduced differentiation and regeneration, an effect not linked to the osmotic potential, but to the high carbon source (Leva and Muleo, 1993). Light quality is also significant for shoot induction, with red light, at 39.11 μ mol/m²/s, being the most effective (Muleo and Morini, 1990). In several cases the organogenic response could be anticipated by observation of a diffuse red pigmentation (anthocyanins) in the callus tissue. Excessive anthocyanin accumulation, sometimes resulting from high light levels, however, can be inhibitory and is correlated with loss of regenerative ability.

Tanaka *et al.* (1997) explored shoot regeneration from fruit galls of *A. polygama*, trying to obtain a strain with consistent biological activity. The regenerated plants were identical to the mother plant.

5.1.3. Haploid recovery

Haploid production through culture of anthers or ovules has not been reported in *Actinidia*, although anther and pollen culture have been attempted (Fraser and Harvey, 1986). Haploids have been obtained only by parthenogenic induction of unfertilized egg cells in *A. deliciosa* (hexaploid), achieved by pollination with lethally irradiated pollen (Pandey *et al.*, 1990). Dose rates of 500, 700 and 1500 Gy, provided by a cobalt source of gamma-irradiation, were reported to yield good results in terms of pollen germination ability and parthenogenic development (Pandey *et al.*, 1990; Chalak and Legave, 1997). Pandey *et al.* (1990) referred to these parthenogenic haploids as triploids, considering the 'haploidization' of the hexaploid genome, and Chalak and Legave (1996, 1997) called them trihaploids. Two plants with half the chromosome number (87) were recovered and unsuccessful attempts were made to double the chromosome number and obtain fertile dihaploids (Pandey *et al.*, 1990; Fraser *et al.*, 1991). Some of the trihaploids obtained by Chalak and Legave, however, spontaneously doubled in chromosome number, while in others efficient chromosome doubling could be achieved by 5 μ M oryzalin treatment combined with adventitious regeneration. Oryzalin, mixed with an autoclaved 0.6% agarose solution, was used: (i) to coat shoots, with no leaves or apex, which were placed on half-strength MS medium without hormones; or (ii) to coat the excised leaves, which were placed on MS medium with 4.6 μ M zeatin. The regenerated shoots were excised after 8 weeks of treatment and rooted spontaneously in hormone-free MS medium. As *A. deliciosa* may be an allopolyploid, the 'diploids' obtained by spontaneous or induced chromosome doubling are not necessarily homozygous for a number of genetic loci.

5.1.4. Triploid recovery

In various *Actinidia* interspecific hybrids, endosperm cultures gave rise to plants with evidence of mixoploidy, although chromosome numbers tended to cluster around tetraploid, hexaploid and octaploid levels (Mu *et al.*, 1990). Huang and Tan (1988) however, reported the recovery of triploid plants, with 87 chromosomes, from cultures of embryoless mature endosperm of *A. chinensis* var. *chinensis* that regenerated via the embryogenic pathway (Huang *et al.*, 1983). Optimal induction was achieved on MS basal medium with 30 g/l sucrose, 13.7 μ M zeatin and 4.5 μ M 2,4-D, for 25 days in darkness at 25–32°C, followed by 1 month under a 12 h photoperiod (850–1200 lux). Embryo germination occurred in the presence of 1.8 μ M zeatin. The morphological characteristics of these plants were intermediate between the diploid var. *chinensis* (2n = 58) and the hexaploid var. *hispida* (now *A. deliciosa*) (2n = 174).

Gui and Mu also regenerated triploid (87 chromosomes) plants from *A. chinensis* endosperm cultures (in Gui *et al.*, 1993). The endosperm, cultured on MS-derived medium with 13.7 μ M zeatin, 2.3 μ M 2,4-D and 400 mg/l casein hydrolysate, produced a callus from which shoots and roots developed. The triploids were transferred to the field and were fertile. The fruits of one triploid clone had excellent flavour, were about 50% the size of the fruit of the mother plant, contained 12.5% of the seed number, had 33% more soluble solids and 33% higher vitamin C content (Gui *et al.*, 1993). From comparisons with other endosperm-derived plants (with chromosome numbers varying from the diploid 58 to the nearly pentaploid 146), there was no correlation between number of seeds or chromosomes and fruit size.

5.1.5. Protoplast isolation and culture

Several *Actinidia* genotypes have been used for protoplast isolation and culture, e.g. *A. deliciosa* 'Hayward', 'Abbott', lines K6, 26, A₁₆N₁, A₁₁B₂; *A. chinensis*, lines A₁₄N₇, A₁₄B₂; *A. arguta* var. *arguta*, var. *purpurea*, and cv. 'Issai' (6x); *A. kolomikta*; *A. polygama*; and *A. eriantha*. Successful plant regeneration, however, was only reported in some cases (Table 1.1.1).

Table 1.1.1.1. Tissues and conditions used in protoplast isolation in *Actinidia* sp. and yielding regenerated plants.

Genotype	Protoplast source and preconditioning	Isolation conditions	Authors
<i>A. deliciosa</i> line no. 26 (incorrectly named <i>A. chinensis</i>)	Calluses derived from leaf and stem (on TCCW medium with 0.23 μ M 2,4-D, 0.44 μ M BA, 2% coconut milk, 10 g/l sucrose, 1 g/l glucose, 0.3 M mannitol, 0.1 M sorbitol)	0.7 M mannitol, 2% Cellulase Onozuka R10, 0.5% Macerozyme R10, 4–5 h	Tsai (1988) (Cai (Tsai) <i>et al.</i> , 1992)
<i>A. deliciosa</i> 'Abbott' (incorrectly named <i>A. chinensis</i>)	Stem-derived cell suspensions (on MS medium with 1 μ M 2,4-D, 1 μ M BA)	0.5 M mannitol, 3 mM MES, 3% Cellulase Onozuka R10, 1% Macerozyme R10, 4 h	Mii and Ohashi (1988)
<i>A. deliciosa</i> 'Hayward' (incorrectly named Hayward by Oliveira and Pais, 1991)	Petiole-derived friable callus (on SH medium with 20 g/l sucrose, 10 g/l mannitol, 4.52 μ M 2,4-D and 0.46 μ M Kin) 0.5% Macerozyme R10, 16 h	0.45 M Mannitol, 3 mM MES, 1.5% Cellulase Onozuka R10,	Oliveira and Pais (1991)
<i>A. chinensis</i> A ₁₄ N ₁₁ <i>A. deliciosa</i> A ₁₆ N ₁	Leaves of micropropagated shoots (on hormone-free half-strength MS medium, with 20 g/l sucrose), pre-conditioned 5–6 days on MS with 0.14 μ M IAA and 6.84 μ M zeatin Cotyledon callus	0.5 M Mannitol, 3 mM MES, 1.5% Cellulase Onozuka R10, 0.5% Macerozyme R10, 16 h	Raquel and Oliveira (1996)
<i>A. eriantha</i>	<i>In vitro</i> cultured seedling leaf (cultured on MS medium with half-strength macrominerals)	0.7 M Mannitol, 2% Cellulase Onozuka R10, 0.5% Macerozyme R10, 16 h 0.45 M Mannitol, 3mM MES, 1% Cellulase Onozuka R10, 0.5% Macerozyme R10, 0.05% Pectolyase Y23, 8 h	Xiao <i>et al.</i> (1992) Xiao and Han (1997) Zhang <i>et al.</i> (1998)

Callus, suspension cultures, leaves or cotyledons of *Actinidia* species were used in combination with various preconditioning treatments and enzyme mixtures to recover regeneration-competent protoplasts (Table 1.1.1) (Fig. 1.1.2).

Calluses established from petioles 3 months after initiation are a good source of protoplasts (Oliveira and Pais, 1991; Oliveira *et al.*, 1991; Table 1.1.1). For isolation, thin slices of callus tissue were incubated in the dark for 16 h, in Petri dishes in CPW salt solution with mannitol, cellulase and macerozyme (Oliveira and Pais, 1991; Table 1.1.1). Protoplasts were purified by filtration through a stainless steel mesh with pores of 100 μm , mixed with half volume of a 0.6 M sucrose solution and distributed in centrifuge tubes with a layer of W5 salt solution. After centrifugation (120 g, 7 min) protoplasts were recovered from the interface, washed twice in W5 solution and recovered for culture at a density of 5×10^6 protoplast/ml of liquid PD1 medium (SH (Schenk and Hildebrandt, 1972) macro- and micronutrients, with MS vitamins, 0.4 M glucose, 97 mg/l methyl ethane sulphonate (MES), 50 mg/l cysteine, 8.1 μM NAA, 0.2 μM 2,4-D, 0.5 μM kinetin, 4.4 μM BA). Half a millilitre of protoplasts in liquid PD1 were laid over 2.5 ml of PD1 medium solidified with 0.6% agarose Sea Plaque, in Petri dishes (5 cm diam.). The cultures were maintained in darkness at 24°C. Fresh liquid PD1 medium (0.2 ml) was added every 2–3 weeks. After 1 month of culture PD1 medium with 0.2 M glucose was added until microcolonies were visible. PD2 (identical to PD1 with 0.2 M glucose, but supplemented with 73 mg/l glutamine and 0.5 g/l folic acid) was added in volumes of 0.2–0.3 ml until the cultures were transferred to diffuse light after 4 months and supplemented with PH1 Glu 30 medium (SH macro- and micronutrients, with MS vitamins, 30 g/l glucose, 97 mg/l MES, 50 mg/l cysteine, 9.1 μM zeatin). Small calluses, greenish in colour, were taken from the original plate and transferred to semi-solid medium E1 Suc20 (50% MS macro- and micronutrients, with MS vitamins, 20 g/l sucrose, 5 mg/l DTT, 0.1 μM IAA, 9.1 μM zeatin) and subcultured every month. Shoot buds were excised and transferred to H medium (half-strength MS macro- and micronutrients, with MS vita-

mins, 20 g/l sucrose, 5 mg/l DTT, 0.1 μM IAA, 2.3 μM zeatin), and shoot growth and rooting were accomplished on H2 medium (identical to H medium but with no growth regulators).

Leaves of *Actinidia* species release mucilage, which complicates protoplast purification. This problem (apparently not found in *A. eriantha* seedlings) can be overcome by dilution of the digested tissue in an equal volume of salt solution (W5) and centrifugation over a sucrose cushion (0.7 M sucrose) (Oliveira and Pais, 1992; Raquel and Oliveira, 1996; Xiao and Hirsch, 1996). Leaf preconditioning (Table 1.1.1) improved the yield of viable protoplasts. Strategies can be used to isolate different protoplast fractions from leaf explants (Raquel and Oliveira, 1996). The protoplast fraction derived from epidermis and leaf veins showed regeneration potential, whereas mesophyll protoplasts regenerated cell walls and divided only occasionally. The culture media and conditions used to regenerate plants were as described above.

In most cases plant regeneration occurred over a long period (8 months for 'Hayward') and required stepwise induction, including the gradual dilution of the osmoticum and a sequence of culture media and growth regulators, to obtain regeneration-competent calluses. With *A. eriantha* seedling leaf protoplasts, however, a plating efficiency of nearly 20% was obtained after 3 weeks on medium with 4.5 μM 2,4-D, without addition of fresh medium (Zhang *et al.*, 1998). Shoots differentiated from regenerated calluses after several subcultures on medium with 2.3 μM zeatin and 0.6 μM IAA.

5.2. Genetic manipulation

5.2.1. Mutation induction and somaclonal variation

Somaclonal variation based on *in vitro* culture procedures has been described only for *A. deliciosa*. Chiariotti *et al.* (1991) reported the detection of isoenzyme polymorphism among plants regenerated from *in vitro* roots of 'Tomuri'. The main strategies and results described (Table 1.1.2), although promising in terms of genetic variation achieved, can be dis-

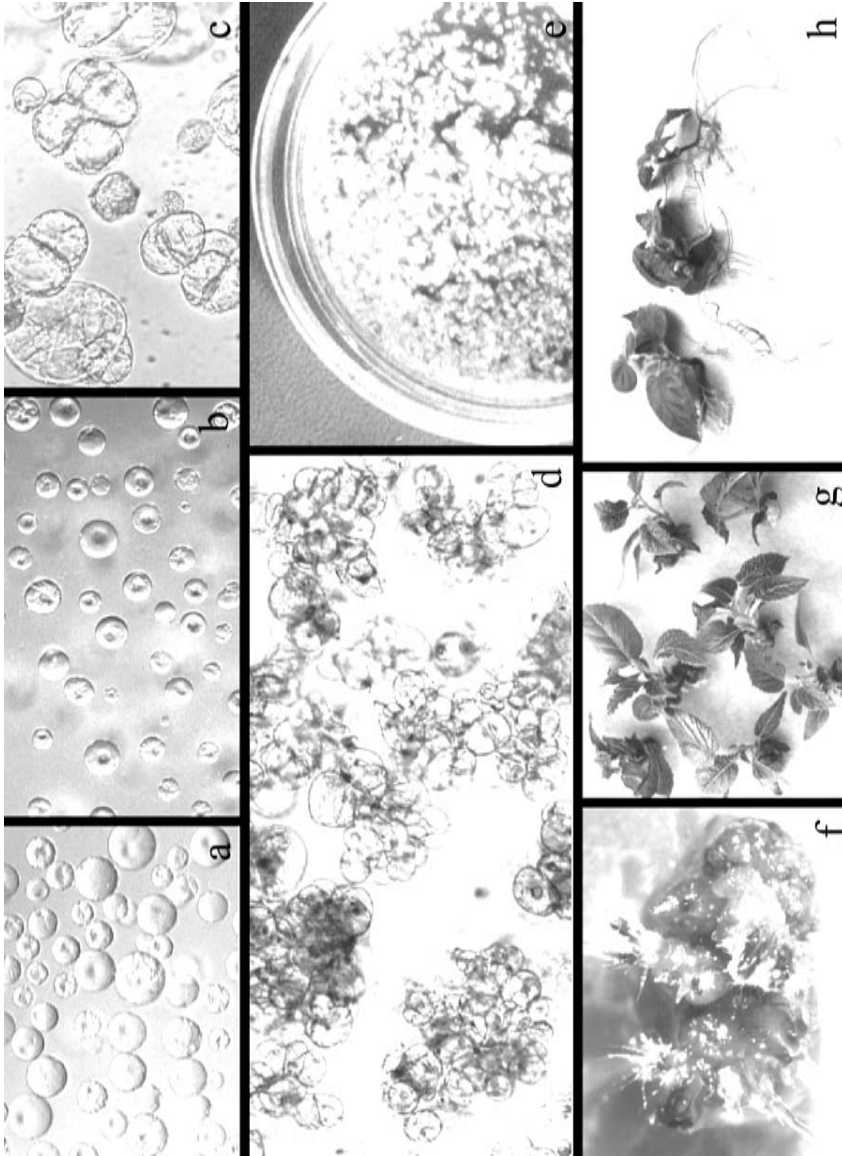


Fig. 1.1.2. Plant regeneration from protoplasts of *A. deliciosa* 'Hayward' isolated from friable petiole hyaline callus. (a) protoplasts after purification; (b) protoplasts at plating density in liquid medium over an agarose-solidified base; (c) first and second divisions observed after 4 weeks in culture; (d) colonies regenerated after 7 weeks in culture; (e) microcalluses after 3–4 months of culture shortly after transfer to light conditions and to zeatin-containing medium; (f) green shoots emerging from organogenic calluses; (g) regenerated shoots prior to transfer to growth regulator-free medium for rooting; (h) rooted shoots at the time of transfer to soil.

Table 1.1.2. Summary of the most important work done on somaclonal variation in *Actinidia deliciosa*.

Goal	Plant and tissue	Strategy (*)	Results	Conclusions	Authors
No specific goal	Leaf and stem of <i>A. deliciosa</i> (**) line no. 26	Protoplast isolation from callus induced from leaf and stem. Plant regeneration in 3 steps: 1) TCCW medium + 0.9 2,4-D/0.54 NAA/2.28-4.56 zeatin 2) TCCW medium + 4.56 zeatin 3) MS medium + 4.56 zeatin Regenerated shoots were rooted or micrografted <i>ex vitro</i>	- Several morphological differences described for leaf shape, size and length of petiole and internodes - Slow growing genotypes observed in the field - Chromosome numbers of 16 somaclones varied between 116 and 180	Micrografted plants can be screened like seedlings for desirable characteristics	Tsai (1988) Cai (Tsai) <i>et al.</i> (1992) Ke <i>et al.</i> (1993)
Obtain somaclonal variation	Leaf blade of <i>A. deliciosa</i> 'Hayward'	Three-step regeneration strategy: (i) Callus induction – 2.26 2,4-D/0.27 NAA (ii) Proliferation – 1.14 IAA/4.44 BAP (iii) Shoot induction – 4.56-9.12 zeatin	- DNA dodecaploid plants obtained (probably by restitutional mitosis), with stomatal guard cells significantly greater than those from hexaploid somaclones - Dodecaploids were more difficult to multiply than hexaploids, but similar in morphology	- A validated method to obtain somaclonal variation - Production of larger fruits is expected from the dodecaploid plants	Boase and Hopping (1995)
Obtain increased tolerance to lime-induced iron chlorosis	Leaves of <i>A. deliciosa</i> 'Hayward' and 'Tomuri'	Callus and shoot induction obtained with 2.68 NAA/4.44 BA, at 3 different pH values: (i) pH 5.7; (ii) pH 7.0; (iii) pH 7.5 Shoot proliferation obtained with 6.65 NAA/0.25 IBA/0.29 GA ₃ , with pH regimes maintained Material amplification achieved in proliferation medium at pH 5.7, Rooting also at pH 5.7	- Higher vigour of somaclones regenerated at high pH - No clear effect of pH on plant tolerance to lime - Some somaclones, able to grow <i>in vitro</i> at high pH, were more tolerant to lime than controls	- The <i>in vitro</i> regeneration and selection methods seem useful to create and select variability in kiwifruit - The methodology is promising to obtain tolerance to high pH and lime levels in soil	Marino and Bertazza (1998) Marino <i>et al.</i> (1998)

(*) Concentrations of growth regulators in μM .(**) Incorrectly named *A. chinensis* in the work of Tsai (1988).

turbing, as simple culture procedures apparently do not avoid producing genetic variation. Gamma-irradiation of axillary and adventitious buds was also attempted for mutation breeding, but further work is needed to optimize treatments and dose rates (Shen *et al.* in Ferguson *et al.*, 1996). The screening methods applied and results obtained did not reveal clearly improved genotypes, but may be further explored with this aim. Measures of the length of stomatal guard cells (Przywara *et al.*, 1988) were very helpful for rapid ploidy evaluations prior to flow cytometry.

RAPD and SSR markers have been used to investigate clonal stability of male and female selections of *A. deliciosa* regenerated *in vitro* (Palombi and Damiano, 2001).

5.2.2. Somatic hybridization

Breeding objectives. As polyploidy and dioecy in *Actinidia* restrict the possibilities of breeding programmes, somatic hybridization may allow the recovery of vigorous and fertile hybrids, permitting breeding and so eventually combining genetic backgrounds within the same gender. Lindsay *et al.* (1995) attempted protoplast fusion to combine *A. deliciosa* with *A. arguta* genotypes, but no regenerants were obtained. They also attempted protoplast co-culture in an effort to produce plants with histogenic cell layers with different genomic backgrounds; however, no chimeric plants were recovered (Lindsay *et al.*, 1995).

Somatic hybridization has been achieved in *Actinidia* (Xiao and Han, 1997), demonstrating the feasibility and potential of the technique. The authors fused protoplasts of *A. deliciosa* with *A. chinensis* (6x + 2x) and *A. chinensis* with *A. kolomikta* (2x + 2x). Regeneration-competent protoplasts of *A. deliciosa* and *A. chinensis* were isolated from cotyledon callus lines (Table 1.1.1), while those of *A. kolomikta*, obtained from the youngest fully expanded leaves of micro-propagated shoots (clone K1), did not regenerate. For isolation of *A. kolomikta* protoplasts the leaves were incubated in a CPW solution with 0.7 M mannitol, 2% cellulase Onozuka R10, 0.5% driselase and 0.1% pectolyase Y-23.

Protocol. Protoplasts of *A. deliciosa* and *A. chinensis*, washed in CPW with 0.7 M mannitol and W5 solutions, were resuspended in W5 (10^6 protoplasts/ml) and mixed in equal volumes. A small volume (0.2 ml) was incubated in a glass tube with an equal volume of a mixture of polyethyleneglycol (PEG) solution (40% PEG 6000, 60 mM CaCl_2 , 0.3 M sucrose), glycine buffer (pH 10.3) and dimethyl sulphoxide (DMSO) (8:1:1) for 10 min. Dilution was slowly performed in W5 solution with 50 mM MES prior to rest (1 h), centrifugation and further dilution. The protoplasts were recovered by centrifugation and cultured. Some modifications were made to the protocol for the hybridization of *A. chinensis* and *A. kolomikta*. The purified protoplasts were mixed and diluted in W5 solution in a ratio of 1:2 and small volumes of the protoplast mixture were placed on a glass dish and allowed to settle for 5 min. Two drops of PEG solution (40% PEG 6000, 60 mM CaCl_2 , 0.3 M mannitol) (1:2) were added to the protoplast mixture and incubated for 5 min before the buffer/DMSO solution was added (W10 solution, prepared freshly by mixing nine parts of stock A – 10% DMSO, 60 mM CaCl_2 , 0.3 M mannitol – with one part of stock B – 1 M glycine-NaOH buffer, pH 10.5) and incubated for 10 min. Dilution steps in W5 with 50 mM MES and rest for 20 min preceded centrifugation and culture as before.

For plant regeneration from the fusion products, a liquid over agarose (0.5%) culture system was used (Xiao *et al.* (1992) incorrectly cited as 1988). For culture they used the NN-69 (Nitsch and Nitsch, 1969) medium, supplemented with 4.5 μM 2,4-D, 1.1 μM zeatin, 200 mg/l casein hydrolysate, 1% sucrose, 0.2 M glucose and 0.4 M mannitol.

Accomplishments. Somatic hybrids were obtained from both fusions and confirmed by RAPD analysis, flow cytometry and intermediate morphology characters, although some results obtained were difficult to explain. Ploidy levels of *A. chinensis* + *A. deliciosa* hybrids were octoploid ($2n = 8x$), and one plant had RAPD banding patterns combining parental banding profiles. Four *A. chi-*

nensis + *A. kolomikta* plants also had a combination of parental RAPD banding profiles, and the regenerated plants were tetraploid ($2n = 4x$), triploid ($2n = 3x$) or pentaploid ($2n = 5x$).

5.2.3. Genetic transformation

Breeding objectives. Various *Actinidia* genotypes, tissues, transformation strategies and gene constructs have been used in transformation protocols, with the main objectives of manipulation of ethylene production (MacDiarmid, 1993; Whittaker, 1997), increased tolerance to drought and diseases (Rugini *et al.*, 1991, 1999; Nakamura *et al.*, 1999) or the accumulation of bioactive compounds important in human health (Kobayashi *et al.*, 1996, 2000). Morphology manipulation was also attempted through the introduction of *rol* genes of *Agrobacterium rhizogenes* or a rice homeobox gene with homology to knotted-1, aiming to increase rooting or regenerate improved rootstocks (Rugini *et al.*, 1991; Yazawa *et al.*, 1995; Kusaba *et al.*, 1999). Direct gene transfer (DGT) and *Agrobacterium* strategies were developed and optimized to recover plants containing and expressing transgenes (see Oliveira and Raquel, 2001, for review).

Most studies on the transformation of *Actinidia* were initially carried out with *A. deliciosa*. Some studies were conducted on *A. arguta* with recovery of transgenic plants, but this species exhibited a higher resistance to kanamycin and required different regeneration protocols (Harvey, in Oliveira and Raquel, 2001). Fraser *et al.* (1995) developed a transformation system for *A. chinensis*. The regeneration of transgenic shoots from *A. chinensis* occurred more quickly than in *A. deliciosa* and with five times as many plantlets produced (Fraser *et al.*, 1995).

Protocol. In DGT to protoplasts (isolated from leaves or calluses), electroporation applied with four rectangular pulses of 200–800 V/cm, 40 μ s each, at 4°C, yielded a very low level of transient expression. A pulse of 200 V/cm was more efficient than PEG 4000 (20%, for 5 min) for plant regeneration and recovery of transgenic shoots

(Oliveira *et al.*, 1991; Oliveira and Raquel, 2001). PEG-mediated permeabilization, however, yielded high levels of transient expression, especially in combination with heat shock (Oliveira *et al.*, 1991). To recover transformants, selection pressure was applied only after regeneration of colonies, maintained for 5–8 months for callus growth and shoot differentiation, and removed to facilitate shoot multiplication and elongation. Under this regime, approximately 80% of the electroporation-regenerated shoots were neophosphate transferase (NPTII) and PCR positive (Oliveira and Raquel, 2001).

Other *A. deliciosa* transformation strategies have been based on the protocols of Matsuta *et al.* (1990, 1993), Kobayashi *et al.* (2000) and Janssen and Gardner (1993). Matsuta *et al.* (1990, 1993) used *in vitro*- or greenhouse-grown material and incubated leaf discs, petioles and stems in a suspension of *Agrobacterium tumefaciens* LBA 4404/pBI121. Co-cultivation was for 3 days on hormone-free medium before the explants were transferred to a MS basal medium containing 200 μ g/ml carbenicillin and 10 μ M zeatin (for 'Hayward') or 5 μ M 4PU (for 'Monty') to regenerate callus and shoots. Kanamycin selection (50 μ g/ml) was imposed immediately or after 18 days. Induced adventitious buds were transferred to MS basal medium supplemented with 0.1 μ M NAA and 2.5 μ M BA, with 50 μ g/ml kanamycin, for shoot proliferation. Root induction on shoots was achieved by culture on MS medium supplemented with 4.9 μ M IBA and direct planting to vermiculite.

Janssen and Gardner (1993) also used *in vitro*-grown material with *A. tumefaciens* strains A281/ or C58/pLAN421 with an incubation time of 1 min. Co-cultivation time was also reduced to 2 days, before *Agrobacterium* elimination (500 μ g/ml cefotaxime) and application of selection pressure (50 μ g/ml kanamycin). Initiation of callus development and shoot induction were achieved using MS medium with 2.3 μ M zeatin and 0.5 μ M NAA solidified with 0.275% Phytigel. Explants were subcultured every 4 weeks to fresh medium with cefotaxime and kanamycin until shoots (2–5 cm) could be rooted on regeneration medium

with 0.6 μM IAA. Rooted plants were transferred to medium with 500 mg/l cefotaxime, without selection pressure, for 1 week prior to transfer to soil.

In the *Agrobacterium*-mediated transformation experiments, acetosyringone (20 or 100 μM), added to the bacterial suspension and to the plant culture medium, was essential to regenerate transformants (Janssen and Gardner, 1993; Matsuta *et al.*, 1993). *A. tumefaciens* strain LBA4404 was also used by Rugini *et al.* (1991), while Uematsu *et al.* (1991) reported the recovery of transgenic shoots from the hypocotyls of 'Hayward'-derived seedlings using *Agrobacterium* EHA101 (A281-derived avirulent strain)/pLAN411 or pLAN421.

Accomplishments. Transformed plants have been grown to maturity, cross-pollinated and their progeny analysed (Fung *et al.*, 1998; Rugini *et al.*, 1999). Fung *et al.* (1998) observed the silencing of the *uidA* gene in some progeny plants and found that none of the actively expressing copies of *nptII* and *uidA* were linked. The stability of *rolABC* genes introduced into pistillate and staminate plants, and transgene inheritance through pollen, was also observed by Rugini *et al.* (1999). The main goals and achievements of *Actinidia* transformation with genes of interest are summarized in Table 1.1.3.

5.3. Cryopreservation

Several strategies have been used for *Actinidia* germplasm preservation. These have included slow growth systems by cold storage (8°C for 1 year) of tips excised from micropropagated shoots (Monette, 1995), and liquid nitrogen storage of stem segments (Jian and Sun, 1989), hypocotyl-derived calluses (Hakozaki *et al.*, 1996) and seedling shoot tips (Susuki *et al.*, 1997).

Cryostorage has been achieved by inserting surface-sterilized 1 cm long stem segments into precooled (0°C) cryoprotectant solution (10% DMSO + 0.5 M sorbitol, or 5% DMSO + 5% glycerol + 5% sucrose) and lowering the temperature to -10°C at 1°C/min (Jian and Sun, 1989). The material was trans-

ferred to -40°C for 3 h, and then transferred to liquid nitrogen. After 120 days of storage, the tissue was thawed at 40°C, washed in MS salts and cultured to dedifferentiate callus and regenerate plants. The regeneration frequency was not affected by cryostorage.

Calluses have been cryopreserved accordingly: culture on medium with 24 or 41% sucrose for 2 days, dehydration twice (20 min each) in 60% and 100% PVS2 solution (30% glycerol, 15% DMSO, 15% PEG, 13.7% sucrose) and immersion in liquid nitrogen (Hakozaki *et al.*, 1996). Frozen tissue was thawed at 37°C, followed by washing in liquid medium with a high sucrose content, and cultured on medium without ammonium nitrate.

For shoot tips, the most satisfactory cryopreservation results were obtained with 2 mm long shoot tips pretreated on media with 37.8 μM abscisic acid (ABA) for 10 days (30% survival), or with 100 μM proline for 1 day (23% survival), and cultured on media with increasing sucrose (0.1, 0.4, 0.7 M) prior to encapsulation in calcium-alginate beads with 0.5 M sucrose (Susuki *et al.*, 1997). Shoot tips were then dehydrated on medium with 1 M sucrose for 16 h, desiccated over silica gel for 8 h, placed in cryotubes and immersed in liquid nitrogen.

Encapsulation-dehydration strategies have been recently reported for routine cryopreservation of *Actinidia* germplasm of various cultivars and species (*A. deliciosa*, *A. chinensis*, *A. arguta*, *A. eriantha* and *Actinidia* hybrids) (Wu *et al.*, 2000; Bachiri *et al.*, 2001). The best results have been obtained when shoots were maintained for 3 months without subculture and cold-hardened for 1 month at 5°C, followed by shoot tip preculture on solid medium with increasing sucrose concentrations (0.5 M, 0.7 M, 1.2 M), before direct immersion in liquid nitrogen.

6. Conclusions

The increasing use of biotechnology in research on *Actinidia* species, in fields as diverse as linkage mapping, reproduction biology, embryo rescue, gene cloning, functional genomics, genetic transformation and

Table 1.1.3. Breeding goals and achievements of *Actinidia* transformation with genes of interest. In all the experiments, *Agrobacterium*-mediated transformation was used.

Genotype	Breeding goals / genes of interest	Achievements / perspectives	Authors
<i>A. deliciosa</i> 'Hayward', and <i>A. chinensis</i>	– Fruit quality improvement through control of ethylene production using sense and antisense ACC oxidase genes	– Antisense ACC oxidase reduced ethylene production in wounded leaves, but only in <i>A. deliciosa</i> – Further studies are required.	MacDiarmid (1993)
<i>A. chinensis</i> 'Earligold'	– Fruit quality improvement through control of ethylene production by use of a construct with ACC synthase and oxidase genes in tandem (in sense and antisense)	– No reduction in ethylene production was observed in wounded leaves, probably due to insufficient ACC oxidase silencing	Whittaker (1997)
<i>A. deliciosa</i> (*) 'Hayward'	– Increased tolerance to drought (through a better root system achieved by <i>rol</i> genes) and reduced susceptibility to <i>Botrytis cinerea</i> (by the <i>osmotin</i> gene)	– <i>rol</i>/ABC transformed plants with dwarf phenotype, reduced flower production, reduced tolerance to winter frost, increased tolerance to drought and reduction of natural resistance to <i>Pseudomonas</i> – Less susceptibility to <i>B. cinerea</i> in <i>osmotin</i> transformed plants	Rugini et al. (1991, 1999)
	– Production of human bioactive peptides in kiwifruit, using a synthetic gene encoding the human epidermal growth factor (hEGF)	– The <i>hEGF</i> gene expressed in young leaves of regenerated plants and highest hEGF content was 65 pg/mg soluble protein	Kobayashi et al. (1996)
	– Increase kiwifruit tolerance to a variety of fungal diseases seriously damaging orchards in Japan (<i>Botryosphaeria</i> sp., <i>Phomopsis</i>, <i>Botrytis cinerea</i>, expressing soybean β-1–3-endoglucanase cDNA	– The possibility of production of bioactive peptides in fruit trees was demonstrated	
	– Investigate the molecular mechanisms underlying morphogenesis in kiwifruit by use of <i>OSH1</i> rice homoeobox gene (homologous to <i>KN-1</i>)	– The gene expressed in young leaves of the transformants, with up to sixfold increase in expression (compared to control plants) – Disease lesions caused by <i>Botrytis cinerea</i> were smaller in transformants than in controls.	Nakamura et al. (1999)
	– Production of resveratrol (a phytoalexin) to achieve increased disease resistance in the plant and also beneficial effects on human health by fruit consumption. Use of <i>Vitis</i> stilbene synthase genes (2 clones tested)	– Some transgenic phenotypes with overexpression of <i>OSH1</i> (driven by 35Spro) were very dwarfed and lacked apical dominance – <i>OSH1</i> acts as morphological regulator – If adapted, dwarfism may lead to labour-saving in kiwifruit vineyard management	Kusaba et al. (1999)
		– The gene was expressed, but resveratrol was always detected in glucosylated form (piceid) in the leaves of the transformants – Piceid had no effect on <i>B. cinerea</i>, but may have some beneficial effect on human health	Kobayashi et al. (2000)

(*) Incorrectly named *A. chinensis* in the work of Kobayashi et al. (1996) and Nakamura et al. (1999).

somatic hybridization, has increased our ability to manipulate species to our advantage in breeding programmes. In the medium to long term, biotechnology should increase the availability of *Actinidia* species or varieties with fruits that are commercially competitive. Technology will also help to ensure the maintenance of genetic diversity through laboratory methods such as cryopreservation. The exploitation of *Actinidia* biological systems for the production of bioactive products important in human health is also promising in the light of preliminary results.

There is still much work needed to provide a better understanding of gene regulation and phenotypic expression, but some of the basic work required to develop these studies has already been accomplished and, while various *Actinidia* genotypes are known to be amenable to *in vitro* manipulation, knowledge now being accumulated will give insight into the methods needed to manipulate other genotypes.

New varieties of *Actinidia*, e.g. 'Zespri™ Gold', which are attractive to farmers and consumers, should end the long period of 'Hayward' monoculture. The genetic diversity available from the regions of origin of this genus and which has recently been integrated into breeding programmes, together with strategies available for *in vitro* manipulation, allows us to anticipate the increasing importance and diversity of *Actinidia* fruits for human consumption.

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2

Anacardiaceae

The *Anacardiaceae* (order *Sapindales*) contains approximately 70 genera and 600 species of mostly tropical origin (Watson and Dallwitz, 1992 onwards). The phytogeographic region, Malesia, which extends from the Malay Peninsula to the Bismarck archipelago to the east of New Guinea, contains more genera within the *Anacardiaceae* than any other region (Bompard and Schnell, 1998). There are several economically important tree species within this family. Cashew (*Anacardium occidentale* L.) and yellow and purple mombins (*Spondias mombin* L. and *Spondias purpurea* L., respectively) are neotropical *Anacardiaceae* species. The kaffir plum (*Harpephyllum caffrum* Bernh. ex K. Krause) and marula plum (*Sclerocarya caffra* Sond.) originated in southern Africa. Mango

(*Mangifera indica* L.), the ambarella or Otaheite apple (*Spondias dulcis* Forst.) and the *Bouea* species have their centre(s) of origin in South-east Asia. The pistachio (*Pistacia vera* L.) probably originated in Iran and central Asia. Many species within the *Anacardiaceae* are exploited for wood and for their secondary products, such as wax and varnish (*Rhus* spp. and *Melanorrhoea* spp.), resins and gums (*Pistacia* spp.) and tanning ingredients (*Mangifera* spp., *Rhus* spp. and *Schinus* spp.). Contact with many species is known to induce severe dermal irritation, e.g. North American *Rhus* spp. (poison ivy and oak) and South-east Asian *Gluta* spp. (rengas). The sap and pollen of several species, including *Mangifera* spp. and *Schinus* spp., can stimulate serious allergic reactions.

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2.1 *Anacardium occidentale* Cashew

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1. Introduction

1.1. Botany and history

The cashew is a small evergreen tree of the neotropics that probably originated in north-eastern Brazil. According to Smith *et al.* (1992), wild populations of cashew trees are restricted to coastal regions and on islands of scrub savannah. The global distribution of cashew cultivation is attributed to the Portuguese, who introduced it to their colonies in Africa (Angola and Mozambique) and Asia (Goa and Cochin on the Malabar coast of India and also Sri Lanka). There are believed to be between eight and 11 *Anacardium* species (Mitchell and Mori, 1987; Nambiar *et al.*, 1990), although the cashew is the only economically important species. Smith *et al.* (1992) indicated that some of the close relatives of cashew, including *A. fruticosum*, *A. giganteum*, *A. microsepalum* and *A. spruceanum*, have edible fruit.

The cashew tree can attain 12 m height and has a spreading dome-shaped canopy (van Eijnatten, 1992). There is a vigorous taproot system, which is thought to provide resistance to drought (Argles, 1976). The cashew tree is polygamous and monoecious with both staminate and hermaphroditic flowers in the panicle inflorescence.

Staminate flowers outnumber hermaphrodite flowers by sixfold or more (Argles, 1976). Trees are generally biennial bearing (Sturtz, 1985). The cashew nut is botanically a fruit, whereas the fruit or apple is the fleshy thalamus or swollen fruit stalk.

1.2. Importance

Cashew is a major fruit and nut crop, with an annual cashew apple production estimated to be 2,033,844 t in 2003 (FAOSTAT, 2004), which is slightly less than cherry (1,796,189 t). Brazil and Madagascar are the leading producers of cashew apple. Annual production of cashew nuts is approx. 1,270,565 t, which is exceeded by almonds (1,447,378 t). The leading producers of cashew nuts are India (450,000 t), Nigeria (176,000 t), Brazil (153,921 t) and Tanzania (130,000 t). Indonesia, Vietnam, Guinea Bissau, Mozambique, Côte d'Ivoire, Thailand and Sri Lanka also have a large production of cashew nuts. World trade of cashew nuts is very important, and has been estimated to be valued at US\$324,002,000 in 1999 (FAO-STAT, 2001). Tanzania, followed by Indonesia and Guinea Bissau, is the leading exporter of cashew nuts. Exports of cashew nuts from Mozambique, which was once the

world leader, have declined as a result of political instability. Cashew oil, also known as cashew nut shell liquid (CNSL), is a valuable by-product of nut processing, and is used as a wood preservative and as a coating for fishing nets.

1.3. Breeding and genetics

The cashew tree, due to the flower structure of the panicle, is primarily an outcrossing species, and consequently is quite heterozygous under natural conditions. Both self-pollination and cross-pollination occur, according to van Eijnatten (1992), but there is strong evidence for self-incompatibility. The flowers are insect-pollinated; however, mechanical pollination is relatively easy. Because of the disproportionate number of male to hermaphrodite flowers and heavy immature fruit drop, yield is quite low. Like many tree species that are not domesticated, there have been many reports that variation among populations of seedlings is minimal. Most production has been from seedling trees; however, high and sustained production from superior vegetatively propagated trees has been documented (Bhaskara Rao, 1998).

According to Smith *et al.* (1992), the cashew was domesticated by indigenous peoples long before the arrival of the Portuguese in Brazil. In recent decades, as cashew production has increased significantly in many countries outside its centre of origin, there has been almost no international exchange of genetic resources, particularly from the centre of origin to other producing nations (Smith *et al.*, 1992). Thus, there may be little genetic gain from breeding programmes when the available genetic resources are so narrow. Nayar (1983) indicated that, despite the large-scale planting of cashew in India over several centuries, a secondary centre of diversity has not developed there, probably because cashew germplasm in India has resulted from only a few introductions. Nambiar *et al.* (1990) attributed the high level of uniformity in seedling cashew trees in India to their narrow genetic base.

Relatively little genetic information is available about the cashew ($2n = 2x = 42$) because there was little breeding effort until relatively recently. In Brazil and possibly Venezuela, the cashew tree appears to be very heterogeneous, although elsewhere, e.g. Africa and Asia, the cashew tree is probably fairly homozygous. The generation period for cashew is only 3–5 years, and hence it should be possible to gather basic genetic information in a committed breeding programme. There are two groupings, which are based upon fruit colour (yellow and red).

1.3.1. Rootstocks

Cashew seedlings have aggressive taproot and feeder root systems, which are thought to confer some level of tolerance to soil stress. Cashew trees can thrive on soils of low fertility and in littoral regions (Sturtz, 1985). Elite cashew selections have been propagated by grafting, air layering and rooting of cuttings, although most orchards consist of seedling trees. There are no soil-borne pests or stresses that cause serious production problems. Although vegetative propagation is used only rarely, there is no production problem that is associated with lack of uniformity of rootstock. Perhaps the most serious disorder associated with roots is damage caused by the root and stem borer *Plocaederus ferrugineus* L. (Coleoptera: Cerambycidae) (Rajapakse, 1998). This is also the most serious insect pest of cashew, and can cause significant crop losses (Rajapakse, 1998). Heavy infestations can cause tree death (Pillai *et al.*, 1976). Bhaskara Rao (1998) suggested that *A. rhinocarpus* and *A. spruceanum*, which are smooth barked, should be tested for their graft compatibility with the cashew tree to provide protection from this pest.

1.3.2. Scions

Major breeding objectives. The potential yield of cashew trees is thought to be ≥ 10 t raw nuts per hectare per annum (Ascenso, 1986a,b). Yields have rarely exceeded 3–4 t raw nuts per hectare when vegetatively propagated trees have been grown under

careful management (van Eijnatten, 1992). More typically, yields in orchards of seedling trees have rarely exceeded 0.4–0.7 t raw nuts per hectare. Therefore there is considerable potential to improve the efficiency of nut production through planting of superior cultivars.

Diseases. According to Freire *et al.* (1995) the most important disease of cashew in Brazil is anthracnose, caused by *Colletotrichum gloeosporioides* Penz. The pathogen attacks leaves, young stems and shoots, floral parts and fruit. Blossom blight (i.e. anthracnose) of cashew is vectored primarily by *Helopeltis antonii* Sign. (Heteroptera: Miridae) (Nambier *et al.*, 1973). As much as $\geq 50\%$ yield losses can be caused by anthracnose, and fruit and nut quality can be significantly reduced (Cardoso *et al.*, 1994). Anthracnose disease is most problematic in growing regions with high rainfall and relative humidity. According to van Eijnatten (1992), control of the disease by periodic systemic fungicide application is possible but uneconomic. The application of elemental sulphur has been utilized to control anthracnose and powdery mildew on cashew, but this can be prohibitively expensive in many growing areas. Moreover, there is evidence that prolonged dusting of cashew with sulphur causes increased soil leaching, lowered pH and increased aluminium in the soil to toxic levels (Majule *et al.*, 1998). Currently, control of anthracnose is achieved by removal and burning of affected plant parts. Other pathogens implicated in blossom blight include *Pestalotiopsis* spp., *Gloeosporium mangiferae* and *Botryodiplodia* spp. (Rajapakse, 1998).

Powdery mildew disease, caused by *Oidium anacardii* Noack, is the leading production problem of cashew in East Africa (Boma *et al.*, 1998b). The disease affects leaves in new flushes, inflorescences, young fruit, nuts and cashew apples. If left uncontrolled, production is lost. Wettable sulphur and Dinocap fungicide are effective for control; however, long-term problems have been associated with the use of sulphur (see above) and Dinocap is too expensive for the main production areas in East Africa.

Dwarfness and precocity. Cashew tree size and shape are highly variable. According to van Eijnatten (1992), canopy surface area determines production in a grove, because flowers and fruit are borne on the outer edge of the canopy. Therefore production can be maximized with small trees or with regular hedging to control tree size. 'Kanaka' and 'Priyanka', both of which resulted from controlled pollinations, are precocious bearers (Bhaskara Rao and Bhatt, 1996).

Insect pests. The tea mosquito *H. antonii* Sign. causes heavy damage to young leaves and inflorescences of the cashew tree (Rajapakse, 1998). Losses that can exceed 30% have been reported in East Africa and India (Nambiar *et al.*, 1990). According to Boma *et al.* (1998a), damage caused by the tea mosquito until recently caused the greatest crop losses in East Africa. Blossom blight, caused by *C. gloeosporioides*, *Pestalotiopsis* spp., *G. mangiferae* and *Botryodiplodia* spp., is associated with damage caused by this sucking insect pest.

Fruit and nut quality. For juice processing, a large cashew apple is desirable. The primary goal of existing breeding programmes has been increased nut yield, nut quality, kernel size and shelling percentage. Nuts with high protein and low sugar levels are preferred. CNSL, a by-product of cashew nut processing, is a mixture of phenol-based monomers, for which there is high industrial demand. CNSL is a resin with a high level of heat tolerance, and is used as a base in paint and varnish and for brake linings. CNSL is highly flammable, is a powerful skin irritant and can be very toxic. One of the constituents of CNSL is cardol, an allergen and irritant. Tree selections have not been made that would have industrial utility, i.e. high levels of CNSL. Conversely, there has been no attempt to select for trees that have nuts with low levels of CNSL. Contamination of cashew nut with CNSL is currently a major constraint of processing.

Breeding accomplishments. Although breeding programmes have existed for two or more decades in many of the major

cashew-growing countries, such as Brazil, India and some African countries, relatively little information is available in the public domain. Lack of movement of cashew germplasm from the centre of origin to cashew-growing countries has severely limited cashew breeding. Results obtained through classical breeding have not been encouraging because of the moderately long juvenile period and a narrow genetic base. Breeding objectives often lack clear definition, and crop improvement has been aimed to meet local needs. For example, high-density planting of improved selections with compact canopies is important in India, and replanting old seedling orchards with compact canopy varieties is preferred (Bhaskara Rao, 1999). Most orchards are still seed-propagated, and vegetatively propagated selections are considered to be uneconomic, as is the use of pesticides and chemical fertilizer inputs. Consequently, cashew production is more akin to agroforestry than horticulture.

Disease and pest resistance. Preliminary investigations have been reported in which sources of resistance to specific insects and diseases have been tentatively described. Millanzi (1998a) determined that damage caused by the tea mosquito is related to the flushing pattern of cashew trees. Early flowering is associated with severe damage. A correlation has been established that relates vigour of the inflorescence bud with resistance to powdery mildew disease (Millanzi, 1998b).

Fruit and nut quality. Breeding programmes to date have concentrated upon increased yield over other nut quality parameters. According to Bhaskara Rao (1998), the narrow genetic base of cashews in many countries limits the potential for cultivar development by conventional breeding. In addition, some of the related species have horticultural characters that could possibly be transferred to cashew. Cashew breeding in India at nine different research centres has resulted in 35 improved cultivars, out of which 24 are selections from openly pollinated seedlings and 11 are derived from controlled pollinations.

2. Molecular Genetics

Molecular studies would be very useful for characterizing the genetic diversity among different cashew cultivars and for identifying genes of commercial interest. This information could be used effectively for cultivar development. Silva Neto *et al.* (1995) have evaluated the use of random amplified polymorphic DNAs (RAPDs) for genetic fingerprinting. Genomic DNA was extracted from seedlings of four dwarf clones, and amplified using six 10-mer arbitrary primers. Twenty-seven amplification products could be used for DNA fingerprinting. Only one primer generated bands sufficient for distinguishing each of the four clones. The results indicated that the RAPD technique is feasible for germplasm analysis and characterization in cashew.

Mneney *et al.* (1998) attempted to assess the level of genetic diversity in cashew genetic resources using RAPD markers. DNA from 25 cashew accessions representing various collections was amplified using random 10-mer primers; however, only limited DNA polymorphisms were observed. Dhanaraj *et al.* (2001) demonstrated that RAPD markers were effective for assessing diversity among 90 accessions from different parts of India. RAPD markers generated by seven Operon primers (10-base-long) were used. Their analysis indicated that the diversity in Indian cashew is not as narrow as reported earlier, but moderate (maximum dissimilarity 63%, minimum 12%). A core collection of 58 selected individuals revealed the same diversity as the entire population. This is an initial step towards efficient germplasm management of cashew in India, and will assist in selection of diverse parents in hybrid breeding programmes.

3. Micropropagation

All of the reports concerning the regeneration of cashew plants from shoot tip and nodal cultures have involved either *in vitro*-germinated or greenhouse-grown seedlings (D'Silva and D'Souza, 1992; Thomas, 1995; Das *et al.*, 1996; Mantell *et al.*, 1998; Ananthakrishnan *et al.*, 1999b; Boggetti *et al.*,

1999; Thimmappaiah and Samuel, 1999). Successful regeneration has been reported from 6–15-month-old greenhouse-grown seedlings (Lievens *et al.*, 1989), 1-year-old greenhouse-grown seedlings (Boggetti *et al.*, 1999), 3-year-old greenhouse-grown seedlings (Leva and Falcone, 1990) and 5-year-old seedlings (Boggetti *et al.*, 1999). Proliferating shoot cultures can be established from highly juvenile material; however, there is progressive decline in shoot initiation with age (Das *et al.*, 1996; Mantell *et al.*, 1998; Boggetti *et al.*, 1999). Although bud break and some shoot elongation occurred with explants from 5-year-old seedlings, axillary bud proliferation and rooting have not been obtained. High phenolic exudation from field-grown shoot tips of cashew is considered to be the major hindrance for *in vitro* regeneration (Thomas, 1995; Das *et al.*, 1996).

Multiple shoot formation from cultured shoot tips and nodes was reported on Murashige and Skoog (MS) (1962) medium, although Boggetti *et al.* (1999) employed MS with half-strength major salts and Lievens *et al.* (1989) used Lepoivre medium. Optimum growth regulator formulations for culture initiation have varied, and this may be a function of genotype or stage of development of the material. Relatively high concentrations of cytokinin(s) have been used, e.g. 5–20 μM benzyladenine (BA) (Boggetti *et al.*, 1999), 22.2 μM BA (D'Silva and D'Souza, 1992) and 2.32 μM kinetin + 9.12 μM zeatin + 4.4 μM BA (Das *et al.*, 1996). Maltose significantly enhances shoot proliferation (D'Silva and D'Souza, 1992; Boggetti *et al.*, 1999). Shoots have been rooted in various ways: (i) inoculation with *Agrobacterium rhizogenes* (Das *et al.*, 1996); (ii) medium supplemented with 2.9 μM indoleacetic acid (IAA) and 4.9 μM indole-3-butyric acid (IBA) (D'Silva and D'Souza, 1992); and (iii) pulsing with 100 μM IBA for 5 days (Boggetti *et al.*, 1999). Rooted micropropagated shoots have survived transfer to soil.

4. Micrografting

Mantell *et al.* (1998), Mneney and Mantell (2001) and Thimmappaiah *et al.* (2002)

described micrografting of microscions from mature trees on to *in vitro*-germinated seedling rootstocks. Nuts can be germinated *in vitro* on semi-solid or in liquid MS (half-strength) medium supplemented with 20 g/l sucrose. Seedlings are decapitated below the cotyledons, and shoot tip and side-grafts (< 0.5 mm to > 5 mm) have been equally successful. The grafted plantlets can be potted out after 10–12 weeks.

Micrografting of seedling shoot tips and axillary buds on *in vitro*-germinated rootstock has also been described by Ramanayake and Kovoov (1999). Scions were treated with IAA, and cut surfaces were irrigated with citric acid solution to control browning. An exogenous auxin (naphthaleneacetic acid (NAA) in culture medium) was required for graft fusion and development.

5. Somatic Cell Genetics

An important prerequisite for the application of somatic cell genetics to cashew improvement is regeneration from elite selections. Srinivasachar (1938), in a comparative study of the morphology of embryogeny within the *Anacardiaceae*, described the development of probable proembryos from the cashew nucellus. He compared these to the adventitious embryos that develop from the nucellus of polyembryonic mangoes.

5.1. Regeneration

5.1.1. Organogenesis

Philip (1984) induced direct shoot organogenesis from (non-elite) cotyledon explants of mature cashew seeds. Lin and Staba (1961) plant growth medium supplemented with 2.85 μM IAA and 2.32 μM kinetin was utilized. It is possible that the *in vitro* growth conditions in this study were suboptimal, because the author described 'bipolar differentiation of organs within the meristematic mound.' Hegde *et al.* (1991) reported organogenesis from embryonic ends of cotyledons from mature seeds. Sections of the cotyledons cultured on Linsmaier and Skoog (LS) medium with NAA and kinetin produced

multiple shoots and roots in 4 weeks. After 2 months of culture, however, further growth of these multiple shoots ceased, and the shoots and roots became desiccated.

5.1.2. Somatic embryogenesis

The first attempts to induce embryogenic cashew cultures involved seed explants (Jha, 1988; Lakshmi Sita, 1989; Hegde *et al.*, 1990, 1991; Sy *et al.*, 1991). Somatic embryos were obtained from cotyledon pieces of 6–8-week-old germinated seedlings (Lakshmi Sita, 1989), cotyledons from mature seeds (Sy *et al.*, 1991) and sections of immature cotyledons (Hegde *et al.*, 1994). Cotyledon explants on medium with NAA, 2,4-dichlorophenoxyacetic acid (2,4-D) and BA formed embryogenic cultures, and somatic embryos developed in the presence of NAA, BA or kinetin (Lakshmi Sita, 1989). In studies by Sy *et al.* (1991), the proximal portion of cotyledons produced somatic embryos when they were cultured initially on Schenk and Hildebrandt (SH) (1972) medium with NAA and BA, followed by culture on medium with casein hydrolysate and adenine sulphate. Hegde *et al.* (1994) observed that cotyledon pieces formed somatic embryos on LS medium with Ca pantothenate, IAA and BA under a 20 h photoperiod. Leafy shoots and roots developed on medium supplemented with activated charcoal; however, there was generally poor organization of shoot meristems.

Ananthakrishnan *et al.* (1999a) and Gogate and Nadgauda (2000) induced embryogenic cultures from (elite) nucellar cultures.

Induction. This protocol is based largely upon the procedures of Ananthakrishnan *et al.* (1999a) and Gogate and Nadgauda (2000). Immature cashew seeds are bisected longitudinally, and each half is placed into culture so that the nucellus is in contact with the plant growth medium. The induction medium of Ananthakrishnan *et al.* (1999a) consists of MS, modified as follows: 60 g/l sucrose, 400 mg/l glutamine, 100 mg/l ascorbic acid, 100 mg/l casein hydrolysate, 8 g/l agar, 20% (v/v) coconut water and 6.78 μM 2,4-D. Somatic embryogenesis from

nucellar tissue has also been reported by Cardoza and D'Souza (2000) on MS medium with 0.5 mg/l picloram. Gogate and Nadgauda (2000) also utilized MS medium, but supplemented with 5.0 μM 2,4-D + 15.0 μM gibberellic acid (GA_3) + 5.0 μM BA. Cultures were maintained at 25°C either with a 16 h photoperiod (40 $\mu\text{mol}/\text{m}^2/\text{s}$) (Ananthakrishnan *et al.*, 1999a) or in darkness (Gogate and Nadgauda, 2000). Embryogenic cultures were evident 7–21 days after explanting.

Maintenance. Gogate and Nadgauda (2000) maintained embryogenic cultures on MS supplemented with 10.0 μM 2,4-D + 15.0 μM GA_3 + 10% coconut water with 40 g/l sucrose and 0.5% activated charcoal. Ananthakrishnan *et al.* (1999a), on the other hand, transferred embryogenic cultures directly into liquid MS medium supplemented with 400 mg/l glutamine, 100 mg/l ascorbic acid, 200 mg/l casein hydrolysate and 4.52 μM 2,4-D for rapid proliferation. Suspension cultures were incubated at 100 rpm on a rotary shaker in darkness.

Maturation. Conditions for development and maturation of cashew somatic embryos have not been well defined. Ananthakrishnan *et al.* (1999a,b) described the development of heart and torpedo stage somatic embryos from embryogenic suspension cultures; however, it is apparent that development beyond this stage was not observed. It would also appear from the figures that somatic embryo development was sporadic. Gogate and Nadgauda (2000) initiated somatic embryo development on MS medium supplemented with 5.0 μM 2,4-D + 30 μM GA_3 + 10% coconut water together with 40 g/l sucrose and 0.05% casein hydrolysate. Somatic embryos developed up to the cotyledonary stage and further germinated when transferred to MS + 0.5% activated charcoal + 3% sucrose + 0.2% Gelrite. The development of cotyledons was, however, negligible as compared to size of cotyledon in a germinating mature zygotic embryo. Complete growth and development of cotyledons of somatic embryos would help in better maturation and conversion rates.

In studies reported by Cardoza and D'Souza (2002) embryogenic callus was formed in presence of 0.5 mg/l picloram. On further transfer to medium with 2.07 μ M picloram + 1.0 mg/l putrescine somatic embryos were formed. Somatic embryos germinated in the presence of 1.89 μ M ABA, whereas Ananthakrishnan *et al.* (1999a,b) obtained reversion of torpedo stage embryos to callus, in the presence of ABA.

Recently, somatic embryogenesis has also been reported using the immature zygotic embryo as an explant (Cardoza and d'Souza 2000; Gogate and Nadgauda, 2003).

In both cases, embryogenesis was direct, where somatic embryos were formed directly from radicle tip/radicle end of the immature zygotic embryo. Somatic embryos were formed in the presence of picloram (2.07 μ M) (Cardoza and D'Souza, 2000) or 5 μ M 2,4-D + 5 μ M BAP + M3 (3 μ M) (Gogate and Nadgauda, 2003). Medium with 20 μ M ABA and 3% maltose was used for maturation and hormone-free medium for germination of somatic embryos (Gogate and Nadgauda, 2003). Cardoza and D'Souza (2000) also reported germination on medium devoid of growth regulators.

5.1.3. Haploid recovery

Although there have been no reports of anther culture and haploid plant recovery, there is a report of *in vitro* germination of cashew pollen (Subbaiah, 1984). Boron was critical for pollen germination and tube growth, and pollen tubes grown in boron-

free medium were distorted in shape. Optimal medium contained 30% polyethylene glycol, 20% sucrose and 20 mg/l each of calcium and boron. These studies may be helpful for devising a medium for anther culture.

6. Conclusions

Despite the importance of the cashew as a fruit and nut crop, there have been few breeding and production advances. This is due to the narrow genetic base outside the centre of origin, barriers that have been erected to discourage the exchange of germplasm and the lack of resources, particularly in Africa. Basic *in vitro* strategies have been developed; however, these have not been refined to the point that, for example, genetic transformation and mutation induction and selection could be used to address specific breeding objectives. With little effort, after screening advanced cultivars for their embryogenic potential, some of the disease problems of cashew (anthracnose, blossom blight, etc.) could be addressed using one or more of these procedures. In the medium and long term, cashew is more likely to be improved by genetic engineering than by conventional breeding.

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2.2. *Mangifera indica* Mango

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1. Introduction

1.1. Botany and history

According to Kostermans and Bompard (1993), the genus *Mangifera* contains approximately 69 species. The centre of diversity for the *Mangifera* is tropical and subtropical Asia from the equator to 27°N latitude. The Malay Peninsula, Borneo and Sumatra, which comprise western Malesia, contain the highest species diversity. Most of the species in the genus are canopy and emergent trees (30–40 m height) of the tropical lowland rainforests. None the less, a few species are found above 1500 m (*M. bompardii*, *M. dongnaiensis* and *M. orophila*). *M. austro-yunnanensis*, *M. indica*, *M. persiciformis* and *M. sylvatica* can be found north of the Tropic of Cancer. Wild mangoes are generally distributed at very low densities on well-drained soils. The trees are evergreen, and flowering is irregular so that fruiting at 2–8-year intervals is typical. Although interspecific hybridization studies have been limited, there appear to be incompatibility barriers that separate many of the species.

The *Mangifera* genus has been subdivided into two subgenera on the basis of morphological characters (Kostermans and Bompard, 1993): subgenera *Limus* (Marchand) Kosterm and *Mangifera*. Species

within subgenus *Limus* are remotely related to mango and appear to be more primitive than species in subgenus *Mangifera*. The *Limus* species are tropical rainforest species of western Malesia, with the exception of *M. foetida*, which is also found in New Guinea, and *M. odorata*, which is known only in cultivation. *M. caesia*, *M. foetida*, *M. kemanga* and *M. odorata* are all widely grown for their fruit in the humid tropics of South-east Asia; *Mangifera pajang* is cultivated on a limited scale in Bali (Bompard and Schnell, 1998).

There are 47 *Mangifera* species within subgenus *Mangifera*, which has been subdivided into five sections: *Marchandora* Pierre, *Euantherae* Pierre, *Rawa* Kosterm, *Mangifera* Ding Hou and a group of species of uncertain position. Section *Marchandora* has only a single species, *M. gedebe*, which grows in areas subject to periodic flooding. The *Euantherae* has three species, *M. caloneura*, *M. cochinchinensis* and *M. pentandra*, which occur in Thailand, Myanmar, Indochina and northern Malaysia in the transition zone between the humid tropics and the drier monsoon climate. All are cultivated for their fruit. Section *Rawa* is not well delimited and contains nine species, of which only *M. griffithii* and *M. microphylla* are cultivated. The species within section *Rawa* generally grow in lowland wetlands in west Malesia.

The common mango is one of 30 species in section *Mangifera*. This section has been further divided into three subsections on the basis of floral structure: pentamerous flowers, tetramerous flowers and an intermediate group. The pentamerous group (14 species) contains *M. laurina*, *M. minor* and *M. sylvatica*, whose fruit bear similarities to the common mango. *M. laurina*, which is highly resistant to anthracnose, is widely cultivated in the humid tropical lowlands. *M. minor* occurs in east Malesia, and grows in dry savannahs and tropical rainforests. *M. sylvatica* occurs at the northernmost range of the genus in northern Myanmar, Thailand and Yunnan. There are 15 species with tetramerous flowers, of which *M. altissima*, *M. torquenda*, *M. magnifica* and *M. quadrifida* are grown for their fruit. The common mango and *M. casturi*, also cultivated for its fruit, are classified within the section having tetra- and pentamerous flowers.

It is probable that the common mango was domesticated independently in different regions of Asia (Bompard and Schnell, 1998), thereby accounting for the two different eco-geographic races of mango that are recognized today: the monoembryonic, subtropical Indian and the South-east Asian, tropical polyembryonic races. *M. indica* is a heterogeneous, outcrossing species with a juvenile period that is approx. 7 years in length. There are pistillate and staminate flowers within the same flower panicle; however, flower opening is asynchronous. According to Mukherjee (1950) the mango is an allotetraploid with $2n = 4x = 40$.

1.2. Importance

Mangoes are currently grown throughout the tropics and subtropics from the equator to approx. 37° latitude. They were spread from their historic centres of cultivation in South and South-east Asia by the Portuguese and Spanish voyages of exploration from the late 15th until the 18th century. The Portuguese most probably transported the monoembryonic mango from their foothold in Goa in India, westward to their colonies in Africa and thence to Brazil. Polyembryonic

mangoes were transported from the Philippines eastward across the Pacific Ocean to Mexico, Central America and South America. The most important mango grown for domestic consumption in Mexico is the 'Manila' (polyembryonic).

World production of mangoes in 2003 was reported to be approx. 25,563,469 t (FAO-STAT, 2004). India is the largest producing country with slightly less than half of the world production (12,000,000 t), followed by China (2,936,522 t), Mexico (1,529,307 t), Thailand (1,350,000 t), Pakistan (937,705 t), the Philippines (931,500 t) and Indonesia (801,777 t). Other large producing countries include Nigeria (729,000 t) and Brazil (605,000 t). International trade is dominated by 'Florida' cultivars, e.g. 'Haden', 'Keitt', 'Kent', 'Sensation', 'Tommy Atkins', etc. Mexico is currently the largest exporter of mangoes (Mukherjee, 1998), and North America and the European Union (EU) are the largest markets for imported fresh mangoes.

1.3. Breeding and genetics

Conventional plant breeding has had relatively little impact on mango cultivar development due to the long juvenile period, polyembryony in South-east Asian types of mangoes and the lack of germplasm resources containing representative genetic diversity in many mango producing countries. Almost all of the commercial monoembryonic cultivars are vegetatively propagated selections made from openly pollinated seedling populations. Indeed, many of the superior Indian mango cultivars, e.g. 'Alphonso', 'Dashehari', 'Langra', etc., are selections that were made in vast orchards of seedling trees that were established at the time of the Mogul emperor Akbar in the 16th century. There is relatively little genetic variation within polyembryonic South-east Asian cultivars, e.g. 'Arumanis', 'Carabao', 'Golek', 'Nam Doc Mai', etc., which are generally propagated from nucellar (clonal) seedlings.

The most significant advance in mango cultivar development followed the system-

atic collection and planting of mango genetic resources in south Florida, USA, by the US Department of Agriculture (USDA) in the late 19th and early 20th centuries. During subsequent years, open pollinations among these accessions, and later among the first and second generation of seedling trees, resulted in significant genetic recombination (Knight and Schnell, 1993) and Florida became a secondary centre of mango diversity. The first generation of 'Florida' mangoes included 'Haden' and 'Keitt', which are seedlings of 'Mulgoba' (sic) (monoembryonic), an accession from India. 'Eldon', 'Glenn', 'Lippens', 'Springfels', 'Tommy Atkins' and 'Zill' are second-generation selections, and are seedlings of 'Haden'. 'Irwin' is a seedling selection from openly pollinated 'Lippens', and is therefore a third-generation selection. The utility of this approach to mango improvement has stimulated similar programmes in Australia, Brazil, Israel, Mexico and South Africa.

Because of the high level of heterozygosity in mango, the difficulty in making controlled pollinations and the high cost and time involved in screening large numbers of hybrid progenies, little is known about the inheritance of horticulturally important traits. Sharma and Majumdar (1988) made systematic observations of >1000 seedling trees following controlled pollinations. They recorded that regular bearing, dwarfness and precocity all appear to be controlled by recessive genes, whereas resistance to mango malformation appears to be dominant. Iyer (1991) observed that yellow flesh colour is dominant over yellow-orange and that internal breakdown is a recessive character. The presence of a beak on mango fruit is a dominant character (Iyer and Subramanyam, 1979).

1.3.1. Rootstocks

Major breeding objectives. Monoembryonic mango cultivars are propagated by grafting on to seedling rootstocks. In many countries, uniform nucellar seedlings are utilized as rootstocks, although this is less common in India. Polyembryonic mango cultivars are grafted only rarely. Ideally, rootstocks can confer resistance to adverse soil

conditions and can affect vigour of the scion. Therefore, superior rootstocks should have certain characteristics (Iyer and Degani, 1998): (i) polyembryony for uniformity; (ii) dwarfing; (iii) tolerance of calcareous soils; (iv) tolerance of soil-borne pathogens; and (v) scion compatibility.

Breeding accomplishments. Israel currently has the only mango rootstock breeding programme; however, none of the selections, either monoembryonic or polyembryonic, are better than the existing '13-1' polyembryonic rootstock that is currently in use (Lavi *et al.*, 1993).

1.3.2. Scions

Major breeding objectives. Mango production is affected by different environmental and biotic factors in various regions. The most important breeding objectives include: (i) regular bearing, particularly in the north Indian cultivars; (ii) dwarfness or compact tree size; (iii) precocity; (iv) attractive fruit, with appealing skin and flesh colour, good taste and flavour, free of fibre and of moderate size (300–500 g); (v) resistance to major diseases, particularly anthracnose, which is caused by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. In Penz.; (vi) resistance to insect pests; (vii) freedom from physiological disorders, such as internal breakdown (jelly seed and soft nose); and (viii) good shipping quality and extended shelf-life (Iyer and Degani, 1998).

Breeding accomplishments. Many of the scion cultivar breeding objectives can be achieved within conventional breeding programmes as was demonstrated by the superior 'Florida' selections that were made within this secondary centre of diversity (Knight, 1998). These cultivars produce fruit with a highly attractive blush and are regular bearers. Some of the 'Florida' mangoes, such as 'Keitt' and 'Tommy Atkins', are also considered to be moderately resistant to anthracnose.

Table 2.2.1 summarizes some of the accomplishments of conventional breeding of mango in India using controlled pollination, as reported by Iyer and Degani (1998).

Table 2.2.1. Mango cultivars that have been released from conventional breeding programmes (from Iyer and Degani, 1998).

Cultivar	Female parent	Male parent	Characteristics
'Mallika'	'Neelum'	'Dashehari'	High TSS, fibreless, high % pulp
'Amrapalli'	'Dashehari'	'Neelum'	Precocious, dwarf, regular bearing
'Sindhua'	'Ratna'	'Alphonso'	Parthenocarpy
'Ratna'	'Alphonso'	'Neelum'	Free of internal breakdown, similar to "Alphonso"
'Au-Rumani'	'Rumani'	'Mulgoa'	Regular bearing, fibreless
'Manjiri'	'Rumani'	'Neelum'	Regular bearing, dwarf
'Neelphonso'	'Neelum'	'Alphonso'	High TSS, dwarf
'Neeleshwari'	'Neelum'	'Dashehari'	High TSS, dwarf
'Neeleshan Gujerat'	'Neelum'	'Baneshan'	High TSS, dwarf

TSS, total soluble solids.

The main breeding goals have been to overcome the problems of alternate bearing and internal fruit breakdown, which are inherent in many of the traditional monoembryonic cultivars of India. Large-scale evaluations of seedling trees following controlled crosses between monoembryonic 'Florida' cultivars, i.e. 'Irwin', 'Sensation' and 'Tommy Atkins' as female parents with 'Kensington Pride' (polyembryonic) are under way at various locations in Queensland, Australia (Whiley *et al.*, 1993). The goal of the Australian programme is to develop new cultivars with greater disease resistance, skin colour, flavour and postharvest performance.

2. Molecular Genetics

2.1. Molecular markers

2.1.1. Protein markers

Genetic relationships among traditional Asian mango cultivars and the Florida mangoes have been addressed using biochemical and molecular markers. Isozymes have been widely used to differentiate among fruit cultivars, to determine parentage of existing cultivars, to characterize seedling populations following controlled pollinations and to construct genetic linkage maps. Gan *et al.* (1981) demonstrated that genetic variation can occur within putative mango cultivars in South-east Asia. Forty-one mango cultivars derived from self- and open-pollinated trees were characterized by Degani *et al.* (1990).

They identified six loci with 17 allelomorphs: PGI: EC 5.3.1.9.; TPI: EC 5.3.1.1; LAP: EC 3.4.11.1; IDH: EC 1.1.1.42; PGM: EC 2.7.5.1; and ACO: EC 4.2.1.3. Using these markers, it was possible to confirm the outcross origin of some mango cultivars and to demonstrate that the putative parentage of other cultivars was inconsistent with isozyme banding patterns. Degani *et al.* (1992) later demonstrated with mango that there were two distinct zones of PGI activity, PGI-1 and PGI-2, and showed that four alleles control PGI-2 banding. Schnell and Knight (1992) were able to differentiate zygotic from nucellar seedlings in populations derived from openly pollinated polyembryonic mango rootstocks using IDH, LAP, PGI, PGM and TPI.

2.1.2. DNA markers

Arumuganathan and Earle (1991) estimated that the nuclear DNA content of mango is only 0.91 pg. Because of their high level of polymorphism, DNA markers have greater usefulness than protein markers. There have been several studies on the use of molecular markers for taxonomic purposes of mangoes: López-Valenzuela *et al.* (1997), using random amplified polymorphic DNAs (RAPDs), concluded that 'Manila', the most widely grown mango cultivar of Mexico, is identical to 'Carabao', the premier mango of the Philippines. Eiadthong *et al.* (2000) studied the phylogenetic relationships among 14 *Mangifera* species using amplified fragment length polymorphisms (AFLPs). Ravishankar

et al. (2000) evaluated the genetic relatedness among several mango cultivars of India using RAPDs. Adato *et al.* (1995) performed a genetic analysis of the progeny of a controlled 'Tommy Atkins' × 'Keitt' cross, and demonstrated that each cultivar and rootstock selection could be identified by distinct minisatellite (i.e. variable number of tandem repeats (VNTR)) markers. Schnell *et al.* (1995) examined 25 mango accessions of the National Mango Germplasm Repository (Miami, Florida, USA) for RAPD genetic markers using 80 10-mer primers. Combinations of commercial primers OPA 15, OPA 18, OPA 19, OPF 12, OPF 13 and OPF 20 could be used to differentiate the 'Florida' mango cultivars (Schnell *et al.*, 1995). Bompard and Schnell (1998) performed an UPGMA cluster analysis of subgenus *Mangifera* based upon the RAPD banding patterns recorded by Schnell and Knight (1992) and Schnell *et al.* (1995) and validated the current taxonomy based upon floral morphology (see above).

Jayasankar *et al.* (1999) utilized RAPDs to detect DNA markers that could be associated with selection *in vitro* of mango embryogenic cultures that were resistant to the phyto-toxin(s) produced by *C. gloeosporioides* (see Section 3.2.1). They observed that distinct markers that were associated with *in vitro* selection occurred in 'Carabao' with eight primers and in 'Hindi' with five primers.

2.2. Marker-assisted selection

Marker-assisted selection (MAS) offers great potential for improvement of quantitative traits in crop plants. There are clear advantages for the use of molecular markers in plant breeding, such as a decreased number of breeding generations, the availability of a uniform method for scoring, no need to use phenotypic scoring until the end and, finally, the possibility for obtaining information on the percentage of the genome contributed by each parent in the offspring. Molecular marker analysis enables the identification of genome segments, the so-called quantitative trait loci (QTL), which contribute to the genetic variance of a trait and thus to the

selection of superior genotypes at these loci without uncertainties due to interaction of genotype with the environment and experimental error. Selecting for favourable QTL effects based on MAS has great potential for improving specific traits.

Although molecular markers have been used for taxonomic purposes with mango (Schnell *et al.*, 1995; López-Valenzuela *et al.*, 1997; Eiadthong *et al.*, 2000; Ravishankar *et al.*, 2000), mango has not been the subject of MAS. It is notable that, although mango has 40 chromosomes, it has a comparatively small haploid genome size, which is only three times as large as the recently sequenced genome of *Arabidopsis thaliana* (the plant with the smallest genome size known), about half that of tomato and comparable to that of rice (Arumuganathan and Earle, 1991). Clearly, the comparatively small mango genome would facilitate the identification of molecular markers and the creation of a genetic map. MAS will become more popular as allele-specific markers become available through genomics research programmes (see Section 2.4).

2.3. Gene cloning

Genetic transformation of mango cultures (see Section 3.2.2) potentially allows the transfer of different genes to manipulate specific processes. Nevertheless, genetic manipulation of any crop requires that relevant genes should be available. Mango has been the subject of molecular studies research for a few years, and genes have been identified that are, for the most part, associated with fruit ripening.

Early studies showed that changes in mRNA and protein content occur during fruit ripening (López-Gómez and Gómez-Lim, 1992). Several different mRNAs (as assayed by *in vitro* translation) increase during ripening of mango fruit. Some mRNAs decrease and some remain constant throughout ripening. At least some of the mRNAs that decline in quantity during mango ripening probably encode photosynthetic enzymes, a situation homologous to tomato fruit, as it is known that during tomato

ripening the photosynthetic apparatus is dismantled (Piechulla *et al.*, 1985). This method for detecting changes in mRNAs has low sensitivity and, therefore, only the most abundant proteins can be detected. Thus, the results of such experiments are preliminary indications of changes in gene expression. For that reason, the next step in the molecular analysis of fruit ripening is the construction of a gene library. This approach has already yielded valuable information with several fruit species, and several genes have been isolated (Giovannoni, 2001). With mango, complementary DNA (cDNA) libraries have been constructed, mainly from ripe fruit, and these libraries have been screened using several approaches. The mRNA for virtually all the ripening-related genes isolated to date have been shown to be absent or at a low level in immature fruit, increasing during maturation and declining as ripening progresses (Giovannoni, 2001).

Mango fruit are highly perishable commodities due to over-ripening, which is mainly caused by the sharp increase in ethylene production and occurs simultaneously with the climacteric peak (Tucker and Grierson, 1987). Mangoes have poor storage quality and technologies for longer-term storage, e.g. controlled or modified atmospheres, are associated with physiological disorders, apart from the fact that there is no clear evidence that mango ripening can be delayed satisfactorily (Chaplin, 1989). Genetic manipulation of mango fruit ripening represents an attractive alternative to extend storage life and, therefore, the isolation of mango genes coding for enzymes involved in ethylene biosynthesis has been a target for research. The two key enzymes in the ethylene biosynthetic pathway are those catalyzing the conversion of S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) and ACC to ethylene, i.e. ACC synthase and ACC oxidase or ethylene-forming enzyme (EFE), respectively (Bleecker and Kende, 2000).

Two cDNA clones that code for mango ACC synthase and ACC oxidase have been identified (Gómez-Lim, 1993). Their expression during ripening was studied in pulp and peel in Northern blot-type experiments.

The ACC synthase message is undetectable in unripe fruit and starts to appear in turning fruit, reaching a maximum in ripe fruit (Gómez Lim, 1993). This pattern of expression is similar in the peel and in the pulp; however, the message appears in the pulp before the peel. The ACC oxidase message shows a similar kinetics in both types of tissue, but the message is clearly detectable before any ACC synthase message becomes detectable (Gómez Lim, 1993). These results suggest that ACC oxidase is expressed before ACC synthase and that ripening starts on the inside of mango fruit and proceeds outwards. Ethylene-treated mango fruits show a different pattern of expression, with ACC oxidase and ACC synthase appearing initially in the peel.

If ethylene is being actively produced, the gas must be clearly perceived by plant cells and therefore ethylene sensitivity must play an important role. The molecular mechanisms underlying ethylene perception and signal transduction are beginning to be understood. Major advances in understanding ethylene signal transduction have come from a molecular genetic approach using ethylene-responsive mutants from *A. thaliana* and tomato, and several genes coding for ethylene receptor homologues have been isolated from those plants (Johnson and Ecker, 1998).

Wilkinson *et al.* (1997) have shown that transgenic tomato plants containing a mutated ETR1-1 from *A. thaliana* exhibit significant delays in fruit ripening and flower senescence and abscission. Therefore, the genetic manipulation of the ethylene receptor represents an interesting alternative to control ethylene production and delay ripening, particularly in those fruits where other alternatives, such as storage in controlled atmospheres, have not been effective. A cDNA coding for a mango ethylene receptor has been isolated (Gutiérrez-Martínez *et al.*, 2001). The message seems to be present at low levels in immature fruit and increases as the fruit ripens. Interestingly, mechanical wounding also seems to up-regulate the expression of the receptor.

The field of volatile compounds released during ripening has received considerable

attention in many fruit. Several studies have convincingly shown that the profile of released volatiles changes as ripening proceeds (Ibañez *et al.*, 1998; Olle *et al.*, 1998; Sakho *et al.*, 1998; Ansari *et al.*, 1999; Saby John *et al.*, 1999; Andrade *et al.*, 2000; Bender *et al.*, 2000). It is likely that many of these compounds, most of which are terpenoids, are determinants of flavour and aroma and many of them originated from the metabolism of fatty acids via the β -oxidation pathway. It is known that several fatty acids, particularly linoleic and oleic acids, decrease in concentration during ripening. In this respect, a cDNA for thiolase, an enzyme from the β -oxidation pathway, has been identified as having a ripening-specific pattern and to be up-regulated during ripening (Bojórquez and Gómez Lim, 1995). A cDNA for acyl-coenzyme A (CoA) oxidase, the key enzyme in the β -oxidation pathway, has been isolated from mango fruit and shown to behave similarly to thiolase (A. Nila-Mendez and M.A. Gómez-Lim, Irapuato, Mexico, unpublished data). These enzymes might be involved in metabolism of fatty acids to produce volatile compounds. Interestingly, the contents of sugars and organic acids can also influence the flavour properties of mango (Malundo *et al.*, 2001).

The manipulation of fruit aroma and flavour is a long-established research goal and, accordingly, the isolation of genes coding for enzymes involved in biosynthesis of these compounds has been targeted. The gene coding for alcohol acyl transferase, an enzyme presumably involved in the synthesis of compounds implicated in fruit flavour, has been identified in mango (GB: AX025510; patent WO 0032789-A 36).

Alternate oxidase is involved in the cyanide-resistant respiratory pathway. It has been studied mainly in thermogenic species, and its activity is correlated with heat production, necessary to volatilize foul-smelling compounds to attract insect pollinators. There is a significant participation of this pathway in the climacteric of many fruit. A cDNA coding for mango alternate oxidase has been isolated and the message has been detected by Northern blot analysis in unripe fruit and shown to increase substantially in

ripe fruit (Cruz-Hernandez and Gómez Lim, 1995). These results have been correlated with similar increases in enzyme activity and protein accumulation. The temperature in ripe monoembryonic 'Alfonso' fruit is up to 10°C higher than in unripe fruit and this has been attributed to the activity of alternate oxidase (Kumar *et al.*, 1990). This extra heat might also serve to volatilize aroma-giving compounds. These results with alternate oxidase were confirmed by Considine *et al.* (2001), who isolated several members of the multigene family of mango alternate oxidase and showed that they were expressed differentially during ripening. They also identified a gene for an uncoupling protein whose mRNA peaked at the turning stage, whereas the protein peaked at the ripe stage (Considine *et al.*, 2001). They suggested a role for alternate oxidase and the uncoupling protein in post-climacteric senescence. Because both mRNA and protein for the alternate oxidase and the uncoupling protein increase in a similar pattern, they hypothesized that their expression is controlled simultaneously.

Ripening of fruit involves a number of metabolic reactions, including synthesis and turnover of the plant cell wall. The latter event is accomplished by a series of hydrolytic, cell wall degrading enzymes that are secreted as ripening proceeds (Fischer and Bennett, 1991). It is interesting that a cDNA clone coding for a homologue of the YPT/Rab class of small guanosine triphosphate (GTP)-binding proteins has been identified from mango and shown to be induced during ripening (Zainal *et al.*, 1996). These proteins appear to control the secretion of other proteins as well as the fusion of membranes in animal cells (Fischer von Mollard *et al.*, 1994). Some of these small GTP-binding proteins have homologues in plants, albeit their role has not been well defined (Staehelin and Moore, 1995). It is tempting to speculate that, based on the pattern of expression, these small GTP-binding proteins facilitate secretion of various hydrolytic enzymes during fruit ripening.

Two additional identified cDNAs code for cell wall hydrolases, i.e. cellulase and β -galactosidase. Mango cellulase increases in

activity in the mesocarp during ripening (M.A. Gómez-Lim, unpublished data) similar to β -galactosidase, which is expressed in ripe fruit at high levels (S. Parra-Arenas and M.A. Gómez-Lim, unpublished data).

In addition to these genes, there are several mango sequences that have been reported in the GenBank. Among them are a genomic sequence for the large subunit of ribulose 1,5-bisphosphate carboxylase (Rubisco) (U39269), and cDNAs for two unidentified clones (AF370123 and AF061639). The pattern of expression of these sequences remains to be determined.

2.4. Functional genomics

The human genome project has been the catalyst for the development of several high-throughput technologies that have made it possible to map and sequence complex genomes. Currently, several bacterial genomes and the genomes of *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *A. thaliana* have been fully sequenced. Nevertheless, the completion of the entire genomic sequence of a particular organism represents the end of the structural genomics segment of the project. It is clear, therefore, that the identification of every gene within the genomes of model organisms is only the initial step for understanding what these genes do and how they interact to make up a living organism. Understanding the functions of the 20,000–50,000 genes comprising plant (and animal) genomes and the variations within a population and their roles in normal development will represent a possibly greater task than the mapping and sequencing efforts currently under way.

Understanding the function of genes and other parts of the genome is known as functional genomics, and research has involved model organisms such as *A. thaliana* and rice. Model organisms offer a cost-effective way to follow the inheritance of genes through many generations in a relatively short time. Functional genomics is characterized by high throughput or large-scale experimental methodologies combined with statistical and

computational analysis of the results. The fundamental strategy in a functional genomics approach is to expand the scope of biological investigation from studying single genes or proteins to studying all genes or proteins at once in a systematic fashion. Computational biology will perform a critical and expanding role in this area: whereas structural genomics has been characterized by data management, functional genomics will be characterized by mining the data sets for particularly valuable information. Functional genomics promises to rapidly narrow the gap between sequence and function and to yield new insights into the behaviour of biological systems. The essential requirement for the implementation of functional genomics is the availability of identified sequences, which can be subsequently used in studies such as high-density arrays. Currently, there are very few sequenced genes from mango, mainly fruit-specific, and an effort to increase this number is needed. Considering the cDNA libraries prepared from ripe fruit in various laboratories around the world, an effort to determine expressed sequence tags (ESTs) would be worthwhile. At the same time, more cDNA libraries from tissues other than fruit are needed. At present, the technology for successful application of functional genomics to mango is well developed, but the raw material, i.e. identified genes, is lacking.

3. Somatic Cell Genetics

3.1. Regeneration

3.1.1. Somatic embryogenesis

The prerequisite for genetic manipulation of mango at the single cell level is an efficient method for regeneration of plants from cell cultures that have originated from the elite or mature phase tree. The embryogenic response of mango is based upon the morphogenic potential of the nucellus. Mango cultivars are either polyembryonic or monoembryonic, depending on their origin (see Section 1.1). The adventitious embryos within polyembryonic mango seeds are

derived from the nucellus, a maternal tissue that surrounds the embryo sac. According to Sturrock (1968), polyembryony in mango appeared to be inherited as a recessive trait; however, more recent studies have demonstrated that polyembryony in mango is under the control of a single dominant gene (Aron *et al.*, 1998). Somatic embryogenesis, which is essentially the same morphogenic response *in vitro*, has been demonstrated to be a dominant trait in other species, e.g. lucerne (Reisch and Bingham, 1980) and cock's foot grass (Gavin *et al.*, 1989), although somatic embryogenesis in red clover has been shown to be conferred by a recessive gene (Broda, 1984).

There are no convenient markers enabling the prediction of the embryogenic response of the nucellus of various mango cultivars. Although early reports suggested that the nucellus of polyembryonic mangoes responded *in vitro* more readily than the nucellus of monoembryonic mangoes (Litz, 1986), this view is no longer accepted. Litz *et al.* (1997) reported that the induction of embryogenic competence in the cultured nucellus of monoembryonic 'Tommy Atkins' was inhibited by the ethylene antagonist aminoethoxyvinylglycine (AVG) and by dicyclohexylammonium sulphate (DCHA), an inhibitor of spermidine synthesis, in contrast to polyembryonic 'Tuehau'. This confirmed an earlier study that demonstrated that somatic embryogenesis in mango was partially mediated by spermidine (Litz and Schaffer, 1987). Therefore, the biosynthesis of ethylene and/or the sensitivity of nucellar tissue to ethylene may be an important determinant for induction of embryogenic cultures from this tissue.

Induction. Induction of embryogenic competence is related to the developmental stage of the nucellus at the time of explanting, and may also be influenced by the physiological condition of the tree (Litz, 1987). Fruits that are approx. 30–40 days after pollination contain seeds in which the nucellus is at the ideal stage for explanting. Mango fruit at the appropriate stage of development are harvested and surface-sterilized with 20–30% (v/v) domestic bleach containing Tween 20

for 30 min. Bleach is rinsed from the fruit with three changes of sterile deionized or distilled water, and each fruit is bisected along its longitudinal axis without damaging the seed under sterile conditions. The immature seed is removed and also bisected carefully along its longitudinal axis. In general, the nucellus can be excised from the immature seed most readily when the embryo (mass) occupies no more than half of the embryo sac. Manzanilla Ramirez *et al.* (2000) obtained optimum results with 'Ataulfo' (polyembryonic), 'Tommy Atkins' (monoembryonic) and 'Haden' (monoembryonic) when the embryo (mass) to immature seed ratio was 1:3. The zygotic embryo (monoembryonic cultivar) or polyembryonic mass (polyembryonic cultivar) is removed and discarded. The nucellus can be removed by carefully peeling it away from the interior of the seed coat using a sterile, flat spatula. After transferring the nucellus on to induction medium in sterile Petri dishes, the cultures are incubated in darkness at 25°C. Thereafter, it is necessary to subculture the explants on to fresh medium at least daily until the oxidation of the explant ceases.

Induction of embryogenic mango cultures from the excised nucellus of immature mango seeds of polyembryonic and monoembryonic cultivars was first reported by Litz *et al.* (1982) and Litz (1984), respectively. Table 2.2.2 includes a list of all mango cultivars that have been established as embryogenic cultures. The efficiency of induction is cultivar-dependent. Litz *et al.* (1998) compared the induction responses of four cultivars, and found that 'Hindi' (polyembryonic) has the highest embryogenic response, followed by 'Lippens' (monoembryonic), 'Tommy Atkins' (monoembryonic) and 'Nam doc Mai' (polyembryonic) in that order. Manzanilla Ramirez *et al.* (2000) compared the induction responses of three cultivars and observed that 'Ataulfo' (polyembryonic) was more embryogenic than either 'Tommy Atkins' (monoembryonic) or 'Haden' (monoembryonic) in that order. Optimization of conditions for induction of embryogenic mango cultures was reported by DeWald *et al.* (1989a) using polyembryonic 'James Saigon' and 'Parris' as

Table 2.2.2. Somatic embryogenesis from nucellar cultures of mango (from Litz and Lavi, 1998; Ara *et al.*, 2000b).

Cultivar	Seed type	Cultivar	Seed type	Cultivar	Seed type
'Alphonso'	Mono	'Heart'	Poly	'Nam doc Mai'	Poly
'Amrapali'	Mono	'Hindi'	Poly	'Neelum'	Mono
'Arumanis'	Poly	'Honc Cambodiana'	Poly	'Ono'	Poly
'Ataulfo'	Poly	'Irwin'	Mono	'Parris'	Poly
'Brander'	Poly	'James Saigon'	Poly	'Peach'	Poly
'Brooks'	Mono	'Keitt'	Mono	'Philippine'	Poly
'Cambodiana'	Poly	'Kensington Pride'	Poly	'Sabre'	Poly
'Carabao'	Poly	'Kur'	Poly	'Simmonds'	Poly
'Chausa'	Mono	'Langra Benarsi'	Mono	'Tommy Atkins'	Mono
'Chino'	Poly	'Lippens'	Mono	'Tuehau'	Poly
'Dashehari'	Mono	'Madu'	Poly	'Turpentine'	Poly
'Everbearing'	Mono	'Manzano'	Poly	'White Langra'	Mono
'Florigon'	Mono	'Mikongensis'	Poly		
'Gedong'	Poly	'Mulgoa'	Mono		
'Golek'	Poly	'Mundan'	Mono		

Mono, monoembryonic; Poly, polyembryonic.

models. The standard procedure has been only slightly modified since then, and utilizes a basal medium consisting of B5 (Gamborg *et al.*, 1968) major salts without $(\text{NH}_4)_2\text{SO}_4$, Murashige and Skoog (1962) (MS) minor salts and organic components, 60 g/l sucrose, 400 mg/l glutamine, 4.8 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.0 g/l gellan gum.

Lad *et al.* (1997) defined the temporal effect of 2,4-D for induction of embryogenic competence in explanted nucellus of 'Carabao' (polyembryonic). Culture initiation required a minimum of 7–14 days exposure to 2,4-D, and a maximum exposure of 56 days. Embryogenic competence of the cultures was optimum after a minimum 28-day exposure to 2,4-D. Nurse cultures consisting of highly embryogenic mango cultures can be effective for stimulating the induction of embryogenic competence from the nucellus of cultivars that are relatively difficult to induce, e.g. 'Nam doc Mai' (polyembryonic) (Litz *et al.*, 1998). Use of a nurse culture involves explanting the nucellus on to sterile filter paper which has been dampened with induction medium and which overlies the highly embryogenic mango culture growing on semi-sterile induction medium. It is not clear whether a nucellar callus is initiated from the explant prior to acquisition of embryogenic

competence (Fig. 2.2.1); however, Litz (1987) demonstrated that somatic embryos can develop directly from the nucellus without an intermediate callus. Very shortly after the appearance of nucellar cultures, they are completely organized, and consist of proembryonal somatic embryos, embryogenic cells, cell aggregates and proembryonic masses (PEMs) (Litz *et al.*, 1993, 1995; Litz and Lavi, 1998).

Maintenance. Embryogenic mango cultures usually appear approx. 30 days after explanting, and are friable and white-to-cream in colour. The cultures rapidly darken on semi-solid medium, and must be subcultured at regular intervals of no greater than 3–4 weeks. PEMs develop from globular somatic embryos in the presence of the primary induction agent, 2,4-D. The PEMs originate as globular embryos, whose development as individual somatic embryos is suppressed by 2,4-D. In suspension culture and to a lesser extent on semi-solid medium, the globular embryos increase in diameter and their protoderm dedifferentiates, thereby becoming embryogenic. Secondary globular somatic embryos develop from embryogenic cells in the protoderm. This highly repetitive or secondary somatic embryogenesis in the presence of 2,4-D is the basis for maintenance of embryogenic cultures.

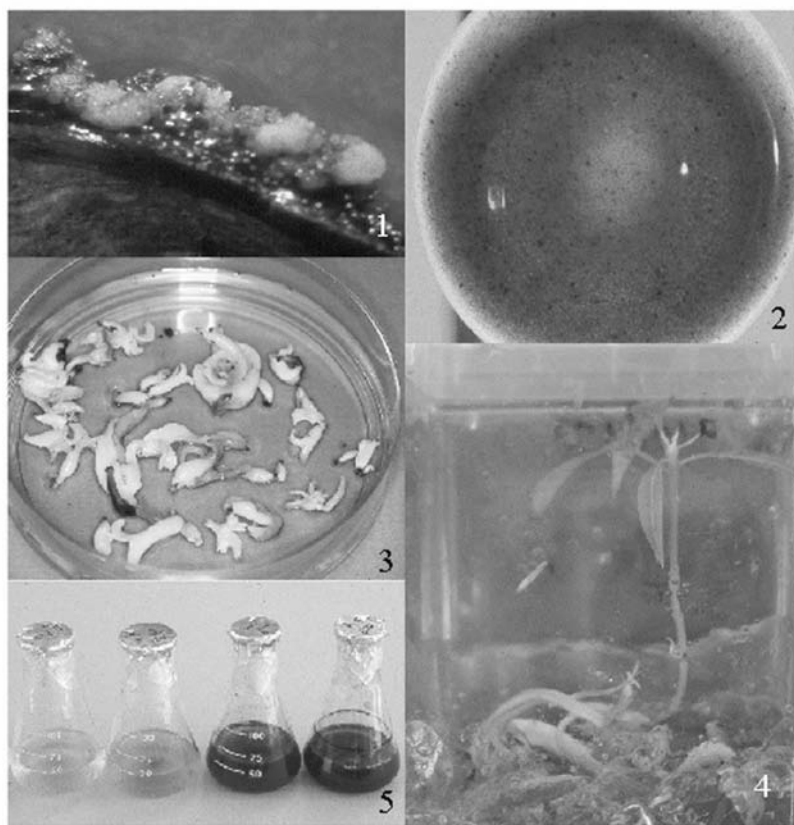


Fig. 2.2. Somatic embryogenesis of mango.

Fig. 2.2.1. Induction of embryogenic culture from mango nucellus. **Fig. 2.2.2.** Maintenance of embryogenic mango cultures in suspension. **Fig. 2.2.3.** Mango somatic embryos developing on maturation medium. **Fig. 2.2.4.** Mango plantlet derived from somatic embryo. **Fig. 2.2.5.** Effect of purified *Colletotrichum gloeosporioides* culture filtrate on embryogenic mango suspension cultures. From left to right: medium control; control; two flasks showing culture filtrate effects.

Embryogenic mango cultures can be maintained either on semi-solid medium or in liquid medium of the induction formulation. Proliferation of embryogenic cultures of many cultivars can be optimized in liquid medium supplemented with $4.8 \mu\text{M}$ 2,4-D (Litz *et al.*, 1984; DeWald *et al.*, 1989a; Fig. 2.2.2); however, rapid proliferation of embryogenic cultures in suspension is a cultivar-dependent trait (Litz *et al.*, 1993). Suspension cultures from embryogenic cultures are initiated by inoculating approx. 300 mg of PEMs into sterile 80 ml maintenance medium in a 250 ml Erlenmeyer flask. The flasks are maintained on a rotary shaker at 100 rpm in semi-darkness at 25°C with regular transfers of PEMs

into fresh medium at 10–14-day intervals. Regular and frequent subculturing is essential to avoid loss of morphogenic potential and darkening of the tissue. A typical suspension culture consists of PEMs, embryogenic cells and multicellular complexes.

Maturation. Development of somatic embryos from embryogenic cultures maintained on semi-solid maintenance medium can occur sporadically and without synchronization, due to the polarity within the tissue culture and lack of direct contact of parts of a culture with the medium containing 2,4-D. Exposure to 2,4-D is necessary for stimulating embryogenic culture proliferation, while at the same

time somatic embryo development is inhibited. It is necessary to transfer embryogenic cultures from maintenance medium formulation to medium without 2,4-D in order to initiate large-scale somatic embryo development. For embryogenic cultures that have been maintained in suspension, the cultures are decanted through filtration fabric with a 1000 μm opening size. The larger fraction is reinoculated into liquid maintenance medium for continued proliferation and the smaller fraction is transferred either into liquid medium or on to semi-solid medium without 2,4-D in order to arrest repetitive somatic embryogenesis and to initiate somatic embryo development.

The plant growth media and conditions for stimulating somatic embryo development and maturation are based upon the protocol described by DeWald *et al.* (1989b) with minor alterations. The initial maturation medium consists of B5 major salts, MS minor salts and organic components, 60 g/l sucrose and 400 mg/l glutamine with or without 2.0 g/l gellan gum. Litz *et al.* (1993) reported that different mango cultivars require different periods for cotyledon differentiation following subculture to maturation medium formulation. Addition of either 4.65 μM kinetin or 4.44 μM benzyladenine (BA) to the maturation medium can stimulate the development of cotyledons and reduce the maturation period (Fig. 2.2.3). Cultures are incubated in darkness at 25°C.

When *in vitro* systems for highly embryogenic cultivars are optimized as suspension cultures, the early cotyledonary somatic embryos that develop in suspension are hyperhydric. Somatic embryos that demonstrate this physiological disorder cannot develop to maturity (Mathews *et al.*, 1992; Monsalud *et al.*, 1995) and become necrotic. Hyperhydricity of early mango somatic embryos can be reversed by partially desiccating heart stage embryos (2–3 mm length) under high relative humidity for 24 h or by plating them on to maturation medium solidified with 6.0 g/l gellan gum (Monsalud *et al.*, 1995). The reversion of hyperhydricity can induce precocious germination of mango somatic embryos, but this can be inhibited by inclusion of 500 μM abscisic acid (ABA) in the maturation medium.

Mango cultivars differ in their response to subculture from liquid maintenance medium, and this must be determined empirically for each cultivar. It is necessary to stimulate 'Hindi' somatic embryo development (cotyledon differentiation) from embryogenic suspension cultures following a stepwise procedure: (i) small fraction (< 1000 μm) from liquid maintenance; subcultured to (ii) liquid maturation media until the early heart stage of somatic embryo development; and subculture to (iii) semi-solid maturation medium. In contrast, it is necessary to subculture the small fraction of 'Keitt' and 'Carabao' from liquid maintenance medium directly on to semi-solid maturation medium.

Mango embryos generally require 4–5 months to develop to maturity *in vivo*, and mature embryos can exceed 6–8 cm length (Fig. 2.2.4). Therefore, the plant growth medium formulations that have been adopted for stimulating growth and development of mango somatic embryos from the heart stage to maturity reflect the differing requirements of these enlarging somatic embryos. The medium that has been utilized for development of mango somatic embryos to maturity consists of B5 major salts, MS minor salt and organic components, 400 mg/l glutamine, 20% (v/v) filter-sterilized coconut water, 40 g/l sucrose and 2.0 g/l gellan gum (DeWald *et al.*, 1989b). The sucrose concentration is gradually reduced to 10 g/l during sequential subculture to fresh media. The cultures are maintained in darkness at 25°C during somatic embryo maturation.

Germination. When somatic embryos begin to germinate, they are transferred to light conditions. The hypocotyl elongates, followed by growth of the tap root. The shoot apex remains underdeveloped until after germination has occurred and, approximately 2 weeks after germination, the shoot emerges (Fig. 2.2.4). Although many mango somatic embryos germinate under these conditions, their survival or conversion is low. Probably the most serious problem associated with low conversion is apical shoot necrosis, a physiological disorder that is

associated with calcium deficiency. Different strategies have been proposed to improve the rate of survival.

1. Litz and Lavi (1998) suggested that the period for embryogenic cultures in/on maintenance medium should be minimal.

2. Ara *et al.* (1998) described the *in vitro* rooting of microshoots obtained from germinated somatic embryos by pulsing them for 24 h with 24.6 μM indolebutyric acid (IBA) in liquid medium followed by transfer to auxin-free medium. Rooting was most efficient in darkness.

3. Enhanced recovery of mango plantlets was described by Litz *et al.* (1993) following the induction of photoautotropism by transfer of small plantlets on to minimal plant growth medium, containing < 5% sucrose and 1% (w/v) activated charcoal. A filter-sterilized air mixture consisting of 20,000 ppm CO_2 in a nitrogen gas carrier was introduced into the growing containers, and the cultures were exposed to a 16 h photoperiod at 180 $\mu\text{mol/s/m}^2$ provided by cool white fluorescent tubes.

4. Early heart stage somatic embryos were encapsulated in calcium alginate containing modified standard mango medium with half-strength major salts and supplemented with 2.9 μM gibberellic acid (GA_3) (Ara *et al.*, 1999). Germination of encapsulated somatic embryos was almost 75% greater than that of non-encapsulated somatic embryos.

3.1.2. Organogenesis

The morphogenic potential of explanted mango cotyledons was first demonstrated by Rao *et al.* (1982), who described the induction of adventitious roots from callus that had been initiated on MS medium supplemented with naphthaleneacetic acid (NAA) and kinetin. Adventitious shoot induction was not observed. Raghuvanshi and Srivastava (1995) successfully initiated shoot-forming callus from fully expanded, young 'Amrapali' (monoembryonic) leaves. Disinfested leaf pieces were cultured in 100 ml liquid MS medium supplemented with 0.05% (v/v) polyvinylpyrrolidone (PVP) as an antioxidant in order to reduce browning of the

leaf tissue. Following transfer at 2 h intervals for approx. 24 h to fresh antioxidant solution and maintained at 75 rpm, leaves were cultured on induction medium, which consisted of MS medium supplemented with 13.0 μM kinetin and 1.1 μM indoleacetic acid (IAA). Induction medium was also the optimum formulation for stimulation of multiple shoot formation. Individual mango shoots were rooted by subculturing them on MS medium with 9.8 μM IBA. Cultures were maintained at 25°C with a 16 h photoperiod provided by cool white fluorescent lights (50–70 $\mu\text{mol/m}^2/\text{s}$). This procedure could be optimized and enable the establishment of morphogenic cultures year-round, in contrast to the embryogenic pathway, which can only be induced during early fruit set.

3.1.3. Protoplast isolation and culture

Ara *et al.* (2000a) described the isolation, culture and regeneration of plantlets from protoplasts isolated from embryogenic suspension cultures of 'Amrapali' (monoembryonic). After approx. 3–4 weeks in suspension, 1 g of embryogenic culture was incubated in 10 ml filter-sterilized growth medium consisting of B5 major salts, MS minor salts and organic components, supplemented with 0.3 M sucrose, 0.4 M mannitol, 0.1 M sorbitol, 2.74 mM glutamine, 1.0% cellulase, 1.0% hemicellulase and 0.5% pectinase (Sigma) with gentle shaking in the dark at 25°C. After incubation for 24 h, the digestion mixture was passed through a stainless steel sieve (50 μm) in order to remove debris. The crude protoplast suspension was centrifuged for 5 min at 100 g, and the supernatant was discarded; this was repeated three times for 3 min for each centrifugation. The pellet was resuspended in 1 ml medium, layered on 3 ml sucrose solution (25% w/v) and centrifuged at 100 g for 7 min. Protoplasts were removed and cultured in the medium formulation as above but modified to contain 0.18 M sucrose, 2.74 mM glutamine and 4.5 μM 2,4-D. Embryogenic cultures were recovered, and PEMs were plated on semi-solid medium. Somatic embryos developed from PEMs following subculture to

medium without 2,4-D, and plantlets were recovered.

The use of protoplast technology in mango improvement is difficult to predict, since the sexual compatibilities of *Mangifera* spp. with the common mango are not known. Although many of the newly described *Mangifera* species have interesting environmental and pest resistance (see Section 1.1), it is not certain if they are isolated genetically from mango. Somatic hybridization could provide a means for genetic recombination of the common mango with some of these species as a way of developing rootstock selections. However, it is unlikely that this approach would have utility for scion development, since the common mango is a tetraploid and the ploidy level of other *Mangifera* spp. is unknown. It is likely that somatic hybridization could result in hexaploids or octaploids, and introgression of useful traits into the common mango would be impossible due to genetic heterogeneity.

3.2. Genetic manipulation

3.2.1. In vitro mutation induction and somaclonal variation

Breeding objectives. In many of the traditional mango producing countries of South and South-east Asia, there is significant consumer resistance to replacement of local mango cultivars by newer selections. Serious production and postharvest problems that have a genetic basis, e.g. alternate and irregular bearing, susceptibility to anthracnose and mango malformation, tree shape and size, etc., cannot be resolved easily using an applied physiology approach. In recent years, paclobutrazol has been applied to alternate bearing mango cultivars to promote flowering in the off-years; however, this has resulted in severe decline in trees that have been treated for successive years. Conventional mango breeding in India, therefore, has focused upon the development of new cultivars that are largely indistinguishable from traditional selections with respect to fruit size, appearance, taste, flavour and overall quality.

Mutation breeding has not significantly

impacted mango cultivar improvement by production of useful off-types of existing selections; however, there is some evidence that somatic mutations can occur naturally in mango on the basis of variation within seed-propagated polyembryonic cultivars. There are reported to be marked phenotypic differences within 'Kensington Pride' (polyembryonic) trees in Australia, and many of the polyembryonic cultivars of South-east Asia, e.g. 'Arumanis', 'Golek', etc., show significant variation, so that different phenotypes of 'Arumanis', for example, have been characterized as 'Arumanis-1', 'Arumanis-2', etc. Litz *et al.* (1993) reported that there was isozyme variation within a nucellar seedling population derived from two polyembryonic cultivars, 'Carabao' and 'Philippine'.

The most important production and postharvest problem of mango in the humid tropics and subtropics is anthracnose, caused by *C. gloeosporioides* (Penz.) Penz. and Sacc. in Penz. The current strategies for control of this disease involve the use of moderately resistant cultivars, including the 'Florida' cultivars 'Tommy Atkins' and 'Keitt', etc., and at least weekly application of fungicides, i.e. benomyl, maneb or mancozeb, from the time of flowering until harvesting (Dodd *et al.*, 1998). This can result in as many as 25 spray applications in a season.

Protocol. The effect of irradiation on embryogenic cultures of different mango cultivars, i.e. 'Hindi', 'Keitt' and 'Tommy Atkins', was reported by Litz (2001). Embryogenic mango cultures growing on semi-solid maintenance medium were exposed to 0–200 Gy irradiation provided by ⁶⁰Co. The median lethal dose (LD₅₀) of 'Keitt' is approx. 125 Gy; the LD₅₀ of 'Tommy Atkins' is approx. 100 Gy; however, the LD₅₀ of 'Hindi' could not be established within this dosage range. The goal of this study is to utilize induced mutations *in vitro* in order to recover useful off-types of existing cultivars.

There is increasing evidence that culture filtrates produced by pathogenic fungi and bacteria can be utilized not only to select for resistance to the pathogen *in vitro* (Hammerschlag, 1992), but also to induce the host resistance response (see Chapter 22). In

order for the former approach to be used effectively, the prerequisite is a highly morphogenic (i.e. embryogenic) suspension culture that can be challenged with the culture filtrate. Litz *et al.* (1991) observed that the culture filtrate of *C. gloeosporioides* could be used as a selective agent in mango suspension cultures that were cultured in maintenance medium formulation (Fig. 2.2.5). Somatic embryos were recovered from embryogenic cells and PEMs that had survived exposure to culture filtrate, and regenerants appeared to show resistance to inoculation with the pathogen. Jayasankar *et al.* (1999) characterized the *in vitro* effects of *C. gloeosporioides* phytotoxin that had been purified according to established protocols (Gohbara *et al.*, 1977, 1978), presumably colletotrichin, and of the crude culture filtrate of *C. gloeosporioides* on the mortality and growth of 'Hindi' and 'Carabao' embryogenic cultures. This study established the LD₅₀ values for the effect of culture filtrate and phytotoxin on embryogenic cultures and the growth curves of challenged cultures. In a later study with the same mango cultivars (Jayasankar and Litz, 1998), embryogenic cultures were either exposed continuously for four cycles of challenge/selection/regrowth or were challenged for one, two, three and four complete cycles with the purified and partially purified culture filtrate of *C. gloeosporioides*. At the end of each cycle, surviving PEMs were cloned and either rechallenged or subcultured on to somatic embryo maturation medium.

Accomplishments. At least three successive challenges with either crude filtrate or purified phytotoxin were necessary in order to stimulate the expression of anti-fungal genes *in vitro*. This was measured by co-culturing the challenged material with a virulent strain of the pathogen. Co-culture of the pathogen with resistant cultures resulted in the suppression of fungal growth, and the anti-fungal properties of the PEMs increased with each cycle of challenge and selection. There was enhanced production of extracellular chitinase and β -1,3-glucanase from selected anti-fungal cultures. An additional chitinase isozyme at 45 kDa was observed

with anti-fungal 'Hindi' and at 25 kDa with anti-fungal 'Carabao', with respect to the controls. There was stable expression of the anti-fungal nature of the resistant lines in suspension cultures and in somatic embryos for more than 2 years after selection. Several RAPD markers were associated with selected cultures that expressed strongly anti-fungal properties (Jayasankar *et al.*, 1998). There was no variation in RAPD markers of the unchallenged controls with respect to the parent trees, indicating that exposure to either the phytotoxin or culture filtrate is essential for anti-fungal expression. These results also demonstrated that embryogenic cultures are stable genetically, and the induced variation does not appear to be a result of somaclonal variation. Furthermore, it seems probable that the phytotoxins themselves are highly mutagenic.

In vitro-induced mutation followed by selection can be a highly efficient method for addressing a specific breeding problem of perennial trees for which there are both an effective selection agent and a highly embryogenic regeneration protocol. Unfortunately, there are relatively few such selection agents that can be utilized in this manner.

3.2.2. Genetic transformation

Genetic transformation is the only practical solution for improving existing elite selections of perennial species for specific horticultural traits. The transformation of mango has been reviewed by Litz and Gómez-Lim (2002).

Protocol. The genetic transformation of mango was first reported by Mathews *et al.* (1992, 1993), who used embryogenic cultures of 'Hindi' and of a 'Keitt' zygotic embryo-derived embryogenic line, respectively. These two studies utilized different disarmed, engineered strains of *Agrobacterium tumefaciens*: (i) strain C58C1 containing the plasmid pGV 3850::1103 with the selectable marker gene for neophosphate transferase (NPTII), which confers resistance to the antibiotic kanamycin (Mathews *et al.*, 1993); and (ii) strain A208 containing the plasmid

pTiT37-SE::pMON9749, a co-integrate vector, with genes for NPTII and the scorable marker β -glucuronidase (GUS or uidA) (Mathews *et al.*, 1992). A subsequent report by Cruz Hernandez *et al.* (1997) utilized *A. tumefaciens* strain LBA4404 containing NPTII, GUS and genes that mediate a horticulturally useful trait (see below) in binary plasmid pBI121. Mathews and Litz (1990) in an earlier, preliminary study had demonstrated that 12.5 μ g/ml kanamycin is toxic to embryogenic suspension cultures, whereas much higher levels (200 μ g/ml kanamycin) are toxic to embryogenic cultures that are grown on semi-solid medium.

All of the reported genetic transformations of mango have followed a similar protocol; however, the most effective procedure has involved a two-step selection (Mathews *et al.*, 1992; Cruz Hernandez *et al.*, 1997). Embryogenic suspension cultures in their logarithmic phase of growth are separated by passing them through sterile filtration fabric (1000 μ m pore size). The large fraction (> 1000 μ m) is gently abraded with a sterile brush and then incubated with acetosyringone-activated *A. tumefaciens* for 3 days in liquid maintenance medium (see above), with subculture into fresh medium at 24 h intervals. The cultures are then transferred on to semi-solid maintenance medium supplemented with 200 mg/l kanamycin and 500 mg/l cefotaxime. After 10 months on this selection medium, the embryogenic cultures are transferred to semi-solid maintenance medium containing 400 mg/l kanamycin. Proliferating cultures are subcultured in liquid maintenance medium containing 100 mg/l kanamycin, and somatic embryo development is initiated by subculture on to semi-solid maturation medium. Mathews *et al.* (1993) reported the recovery of transgenic mango plants derived from a 'Keitt' zygotic embryo embryogenic culture, which had been transformed with pGV 3850::1103 containing the selectable marker *nptII*. Genetic transformation was confirmed by: (i) growth in selection medium containing inhibitory levels of kanamycin; (ii) positive histochemical reaction for GUS with X-GLUC (Jefferson, 1987); and (iii) Southern hybridization.

Accomplishments. Loss of mango fruit due to premature ripening and spoilage in storage and *en route* to markets accounts for a significant percentage of total production in most developing countries that have poorly developed infrastructure, i.e. cold storage facilities, poor roads, unreliable transportation, etc. Mango has also become an important export commodity for several developing countries. Extended shelf-life and absence of physiological disorders that cause internal breakdown of fruit, e.g. 'soft nose' and 'jelly seed', of the most important export cultivars, e.g. 'Tommy Atkins', are potentially very important, therefore, for the valuable export trade and for domestic markets.

The mango is a climacteric fruit; consequently, ethylene is a critical regulator of the biochemical processes that occur during ripening. Certain of the rate-limiting genes that mediate ethylene production in mango have been cloned (see Section 2.3). Cruz Hernandez *et al.* (1997) described the genetic transformation of embryogenic 'Hindi' mango cultures with mango ACC oxidase, ACC synthase and ACC alternative oxidase cloned in the antisense orientation and under the control of the 35S constitutive promoter in the pBI121 binary vector. The constructs were transferred individually into *A. tumefaciens* strain LBA4404 by electroporation. Embryogenic cultures were transformed by the two-step procedure described above. Although the phenotype of the transformed lines was not reported, the genetic transformations were confirmed in each case by the GUS reaction, growth in the presence of inhibitory levels of kanamycin, Southern blot hybridization and *NPTII* amplification by PCR. Successful regeneration of plants and inhibition of ethylene production by mature mango fruit could resolve the production problem of premature ripening (jelly seed) and postharvest loss due to spoilage.

4. In Vitro Storage

Mango embryos are considered to be of the recalcitrant type, i.e. they cannot tolerate desiccation during maturation and they do not undergo developmental arrest. Lacking a

period of developmental arrest, mango embryos develop to maturity and then germinate in an uninterrupted sequence. As a consequence, mango seeds (and embryos) cannot survive for more than 3 or 4 weeks under *in vitro* minimal growth storage conditions (Parisot, 1988) or in a conventional seed bank. Monsalud *et al.* (1995) demonstrated that 4–5 mm somatic embryos (late heart stage) could be partially desiccated and stored dry in Petri dishes without plant growth medium for > 30 days without any loss of viability; however, larger somatic embryos could not survive this treatment. This observation has implications for future studies that might focus on developing the concept of an ‘artificial seed’ (i.e. somatic embryo) gene bank for vegetatively propagated tropical fruit trees.

Manipulating mango somatic embryos in order to induce developmental arrest has been investigated. ABA is associated with initiation of developmental arrest and desiccation of orthodox type seeds and embryos and is effective *in vitro* at relatively low concentrations for somatic embryos of the orthodox type (Bewley and Black, 1985). Pliego Alfaro *et al.* (1996a,b) were able to arrest late heart stage mango somatic and nucellar embryo development, respectively, by incorporating high levels of ABA (minimum 100 μ M) in the growth medium. This strategy was effective for arresting growth and development for several months; ABA had a strong residual effect on mango somatic embryo growth inhibition, which persisted for approx. 1 month after subculture of somatic embryos on to medium without ABA. Increasing the osmolarity of the plant growth medium also inhibited somatic embryo development; however, there was no residual effect following the subculture of somatic embryos on to semi-solid medium without osmoticum.

Cryopreservation of embryogenic cultures of mango using different procedures has been demonstrated to be feasible (Wu *et al.*, 2003; Rajani Nadgauda and Pamela Moon, Homestead, Florida, USA, personal communication). Wu *et al.* (2003) compared three cryopreservation protocols for embryogenic ‘Zihua’ cultures: encapsulation–dehy-

dration, pregrowth–dehydration and vitrification. The encapsulation–dehydration procedure was unsuccessful, and only limited survival (8.3%) was obtained following desiccation of PEMs for 1 h to 58.5% moisture content prior to freezing in liquid nitrogen. Vitrification, involving treatment of PEMs with plant vitrification solution 2 (PVS2) (Sakai *et al.*, 1991) for 20 min prior to freezing in liquid nitrogen, was successful (94.3%).

Embryogenic ‘Hindi’ cultures have also been introduced into cryogenic storage (Rajani Nadgauda and Pamela Moon, Homestead, Florida, USA, personal communication) and somatic embryos have been recovered from these cultures. Two procedures were followed: (i) stepwise cooling, in which cryoprotected (5% dimethyl sulphoxide (DMSO) and 5% glycerol) embryogenic cultures were cooled in ‘Mr Frosty’ containers at the rate of $-1^{\circ}\text{C}/\text{min}$ from room temperature to -75°C , followed by rapid cooling to -196°C ; and (ii) rapid cooling (vitrification).

Following the removal of cryovials from liquid nitrogen and rapid warming to room temperature, cultures were thoroughly washed with maintenance medium and plated on semi-solid maintenance medium formulation. Somatic embryo development was initiated by subculturing the PEMs on somatic embryo maturation medium.

5. Conclusions

Cell culture and regeneration systems for several different mango cultivars have been elaborated and confirmed by several groups. Increasing the conversion frequency is essential for certain types of applications. Some of the major breeding objectives can be addressed either by *in vitro* mutagenesis and selection (e.g. anthracnose) or by genetic transformation (e.g. control of fruit ripening), using techniques already described (*in vitro* selection and genetic transformation) with genes that have been cloned from mango. It is clear that directed qualitative improvement of existing mango cultivars can only be accomplished through genetic transformation. Control of fruit

quality, elimination of physiological disorders of certain selections (internal breakdown of fruit), alteration of tree architecture and greater resistance to insect pests and diseases should be the major objectives for genetic engineering of mango. Cryogenic storage appears to be a promising tool to provide a backup for large *ex situ* plantings of clonal mango genetic resources and for experimental materials.

Increasing world trade of fresh mangoes is beginning to seriously impact production in both Israel and the USA, which for many years have provided a major component of

research in biotechnology of this species. The major producing countries, i.e. India, China, Mexico and elsewhere, must invest more heavily in the biotechnologies. Breeding programmes must become more reliant on molecular tools, including map-based breeding, and on the use of genomics to identify gene function.

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2.3 *Pistacia vera* Pistachio

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1. Introduction

1.1. Botany and history

The pistachio tree is small, up to 10 m, and spreading. Leaves are compound-pinnate, hairy when young, later glabrous; they are alternate, deciduous or evergreen, pinnate, more rarely trifoliate or simple. The fruits are semi-dry drupes with edible seeds. They have an external, fleshy hull that loosens from the nut at maturity, but must be removed either by hand or mechanically. The tree requires long, hot summers (mean temperatures $\geq 30^{\circ}\text{C}$ for 98–110 days) and moderate winters with at least 1000 hours $\leq 7^{\circ}\text{C}$ (Ayfer, 1990). Trees thrive in fine sandy loams, but can tolerate saline, alkaline and calcareous soils.

Zohary (1952) recognized 11 species within the genus *Pistacia*, of which six are native to Turkey (Davis, 1965; Yaltirik, 1967). Communities of pistachio are very heterogeneous, their flowering behaviour depending on their location, which can be steppe-forests, steppe or semi-deserts. Floral bud differentiation occurs during the calendar year prior to blossoming (Ayfer, 1963). In the northern hemisphere, shoot elongation begins at the end of March and terminates between the end of April and the middle of May (Crane and Iwakiri, 1981). Generally,

one or two axillary buds located distally on the new growth are vegetative. They are considerably smaller than the inflorescence buds, and may give rise to lateral branches in the following year, or they may remain dormant. Inflorescence buds begin to expand at the end of the following March, and anthesis generally occurs in late May and for about 3 weeks thereafter their growth and differentiation are rapid (Ayfer, 1963). Thus, pistachio bears its fruit laterally on wood produced the previous season (Crane, 1984).

Both staminate and pistillate inflorescences are panicles, which may consist of 100 to several hundred individual flowers. Both types of flowers are apetalous, and they are wind-pollinated. Staminate and pistillate cultivars having similar flowering times must be planted together in orchards to ensure adequate pollination. Fruit maturity is manifested by a change in the epicarp (skin) from translucent to opaque, and a softening and loosening of the epicarp and mesocarp (hull) from the endocarp (shell), which encloses the embryo (kernel) (Crane and Iwakiri, 1981). The endocarp has a thin red-violet fleck and the seeds range in colour from light to dark green. Endocarp dehiscence is first noted along the ventral suture in late July, about the time ultimate kernel size is attained, and progresses along both sutures until physiological maturity (mid-

September). Physiological maturity is signalled by easy separation of the epicarp (hull) from the shell (Crane, 1978).

According to Vavilov (1951) the centre of origin of pistachio includes: (i) central Asia, including north-east India, Afghanistan, Tajikistan and Uzbekistan; and (ii) the Near East, including Asia Minor, the Caucasus, Iran and the mountains of Turkmenistan. Recent archaeological evidence in Turkey, Jordan, Syria, Iraq and Iran indicates that the nuts were being used for food as early as 7000 BC (Kirkbride, 1966; Bender, 1975; Kramer, 1982; De Conteson, 1983). The Queen of Sheba during her visit to Assyria is said to have commandeered the limited crop of nuts for her exclusive use and that of her guests (Whitehouse, 1957). Pistachio trees were planted in the gardens of King Merodach-Baladan of Babylon around the 8th century BC (Brothwell and Brothwell, 1969). In the 2nd century BC, Nicander found pistachios in Susa, a village in south-western Iran close to the border with Iraq (Joret, 1976). In the 1st century BC, Poseidonius recorded cultivated pistachios in Syria (Joret, 1976), and they were probably introduced from Anatolia and thence into Italy in the 1st century AD (Banifacio, 1942; Moldenke and Alma, 1952). Pistachio was introduced into the USA in 1853–1854 (Lemaistre, 1959). Pistachio cultivation has also spread eastward from its centre of origin and was reported in China around the 10th century AD (Lemaistre, 1959). More recently, its culture has begun in Australia.

The word pistachio appears to derive from the Zendor Avestan (ancient Persian language) *pista-pistak* (Joret, 1976). According to Dioskurides, the word pistachio is derived from *pissa* = resin and *aklomai* = to heal, i.e. a plant with wholesome resin.

1.2. Importance

World commercial production of pistachio nuts increased eightfold from approx. 52,000 t in 1976 to 541,957 t in 2003 (FAOSTAT, 2004). Output from Iran, Syria, Turkey and Greece increased 4.7-, 8.2-, 30- and 3.4-fold, respectively, and production in the USA rose from

negligible in 1976 to 52,620 t in 2003, making it the second world producer after Iran.

1.3. Breeding and genetics

Due to cross-pollination, pistachio is very heterogeneous. Breeding strategies exploit this genetic variation using seed orchards and controlled pollination. Because pistachio is dioecious, it is impossible to simply combine the best characteristics of two cultivars, female and male, by hybridization (Barghchi and Alderson, 1989). Typically, only a few cultivars are grown in any pistachio-producing region. The California pistachio industry relies almost exclusively on two seedling selections, 'Kerman', a female cultivar, and 'Peters', a male cultivar (Dollo *et al.*, 1995). Progress has been slow, and many selections are under evaluation (Whitehouse, 1957; Maggs, 1982; Avanzato *et al.*, 1987; Vargas *et al.*, 1987).

1.3.1. Rootstocks

Elite pistachio selections are vegetatively propagated by grafting on to seedling rootstocks. Male and female scions are often grafted on to a single rootstock. Rooting of stem cuttings from mature trees is difficult (Al Barazi and Schwabe, 1982). In addition to *P. vera*, there are nine species that have been used as rootstocks for pistachio: *P. atlantica*, *P. chinensis*, *P. integerrima*, *P. khinjuk*, *P. lentiscus*, *P. mutica*, *P. palaestina* and *P. terebinthus* (Barghchi and Alderson, 1989). *P. atlantica*, *P. integerrima* and *P. terebinthus* are the main rootstocks in California, USA, as they can tolerate most soil-borne fungi and nematodes (Michailides *et al.*, 1988). *P. mutica* and *P. khinjuk* are commonly used as rootstocks in Iran. In Turkey, rootstocks are either wild pistachio species that are grafted *in situ* or pistachio seedlings.

Major breeding objectives. Several soil-borne fungi attack the pistachio. The most serious fungal disease is *Verticillium* wilt, which can quickly kill trees of varying ages (Duke, 1989). Trees are also sensitive to the

oak root fungus, *Armillaria mellea*. The breeding objectives of rootstocks include: (i) resistance to nematodes, soil-borne fungi and saline soil; (ii) vigorous growth; and (iii) selection based upon nursery performance.

Breeding accomplishments. Pistachio rootstocks have been identified with resistance to nematodes and soil-borne fungi. Most pistachios are now grafted on to *Verticillium*-resistant *P. integerrima*, *P. atlantica*, *P. terebinthus* and *P. atlantica* × *P. integerrima* seedlings (Ferguson and Arpaia, 1990). In the USA, 'Kerman' (pistillate) and 'Peters' (staminate) are normally budded on to *P. atlantica* or *P. integerrima* rootstocks (Hall, 1975; Joley, 1979; Crane and Iwakiri, 1981), which are resistant to nematodes and *Verticillium*. In Turkey, Iran and elsewhere in the Middle East, *P. vera* is used as a rootstock for new orchards. Seedlings of *P. vera* produce more lateral roots and thicker stems than the other species and scions can reach budding size earlier (Ayfer et al., 1990); however, there are growth differences among seedlings, resulting in stock–scion incompatibility, which requires intergrafting. Seedlings of *P. atlantica* and *P. khinjuk* elongate rapidly and produce thinner seedlings than other species so that budding size is reached later; however, there is no stock–scion incompatibility between *P. vera* and these rootstocks. Seedlings of *P. atlantica* and *P. terebinthus* are widely used as rootstocks for commercial production (Joley and Opitz, 1971; Woodroof, 1979; Hartman and Kester, 1983). Top-worked cultivars on these two rootstocks outgrow and outperform those grafted on to *P. vera* despite their initial slow growth in the nursery (Joley, 1979). *P. atlantica* and *P. terebinthus* are highly susceptible to *Verticillium* (Hartman and Kester, 1983). Because of its very slow growth habit, *P. terebinthus* produces dwarf trees that bear early, with large and high-quality fruits. *P. terebinthus* and *P. vera* can be suitable rootstocks for intensive cultivation under irrigation (Ayfer et al., 1990). Seedling progenies of *P. vera* × *P. atlantica* and *P. vera* × *P. interregima* are highly resistant to root knot nematode compared to *P. vera* × *P. terebinthus* seedlings,

which are the least resistant (Joley and Whitehouse, 1953); however, *P. terebinthus* has been reported to be tolerant of *Phytophthora* spp. (Pontikis, 1977).

1.3.2. Scions

Major breeding objectives. Pistachio nuts are classified according to nut characteristics, i.e. size, pericarp splitting (dehiscence) rate and kernel colour (Ayfer, 1990). In addition to nut characteristics, trees are selected for pollen production, flowering time, yield quality and potential, uniformity of cropping (to avoid alternate bearing and empty seeds), early cropping and tree form. The main breeding objectives include tree vigour, age of bearing, growth habit and height at maturity, together with characteristics such as flowering time, early cropping, disease resistance, salt tolerance, tree form for mechanical harvesting and higher nut yield and quality.

Breeding accomplishments. Pistachio breeding programmes have been initiated to develop new cultivars only recently. Dioecy represents an inconvenience since the reproductive maturity of pistachio requires 5–8 years (Hormaza et al., 1994). Considerable variation has been reported in wild populations of *P. vera*; Zohary (1952) and Maggs (1973) have listed > 50 pistachio cultivars. Exporters in Iran often supply pistachio nuts from approximately 30 different varieties (Barghchi and Alderson, 1989). 'Ohadi' is the main variety in Iran, accounting for 80% of nut production (Barghchi, 1982). 'Kellegouchi', 'Sedifi', 'Vahidi', 'Mümtaz' and 'Kerman' are Iranian cultivars that are being field-tested in south-eastern Turkey (Kuru et al., 1990). 'Antep' is the main cultivar in Turkey and accounts for > 90% of nut production, followed by 'Siirt', which is grown under non-irrigated conditions. In California, USA, production is almost entirely based on 'Kerman' and the male scion cultivar 'Peter's' under irrigated conditions (Ferguson and Arpaia, 1990). A sister seedling of 'Kerman', 'Lassen', is also grown in California, USA, and produces good-quality, large-sized nuts.

2. Molecular Genetics

There have been only a few studies that have targeted intra- and interspecific genetic relationships, patterns of inheritance and breeding histories. These have traditionally been based on morphological, physiological and biochemical data (Zohary 1952; Caruso *et al.*, 1988). Loukas and Pontikis (1979) and Ettori Barone and Francesco (1996) attempted to correlate morphological traits with isozyme markers to distinguish species and cultivars. The major limitation of isozymes as markers is the lack of sufficient polymorphism among closely related cultivars (Dollo *et al.*, 1995). Attempts to identify DNA markers for distinguishing between male and female plants in several dioecious species have been reported (Durand and Durand, 1990). A random amplified polymorphic DNA (RAPD) technique has been used to identify markers linked to sex determination in pistachio (Hormaza *et al.*, 1994, 1998). The OPO08₉₄₅ marker can be used to screen the gender of pistachio seedlings long before they reach reproductive maturity, resulting in considerable savings of time and resources.

3. Micropropagation

Hansman and Owens y de Novoa (1986), Barghchi and Alderson (1989) and Onay and Jeffree (2000) have reviewed the *in vitro* requirements of pistachio shoot tip and nodal culture. All *in vitro* studies involving pistachio have been summarized in Tables 2.3.1 and 2.3.2. The initiation of cultures from adult material has usually involved pruning, grafting and application of benzyladenine (BA) and gibberellic acid (GA₃) as spray treatments to stimulate new growth of shoots (Barghchi and Martinelli, 1984; Barghchi and Alderson, 1989; Gonzales and Frutos, 1990). Partial rejuvenation of mature materials has been attempted by micrografting mature scion shoot tips on to juvenile *P. vera* rootstocks (Barghchi and Martinelli, 1984; Barghchi, 1985); however, only slow growth of the scion occurred. Pistachio micrografting has been investigated *in vivo* as well as *in vitro* (Abousalim and Mantell, 1992), but rejuvenation was not been obtained. Barghchi and

Alderson (1983b) reported caulogenesis from shoot tips and nodal bud segments (5–8 mm long) excised from aseptic seedlings of pistachio and cultured on Murashige and Skoog (MS) (1962) medium supplemented with 17.8 μM BA and 1.35 μM naphthaleneacetic acid (NAA). Seeds and cultures were incubated at $25 \pm 1^\circ\text{C}$ under a 16 h photoperiod at $3 \pm 1 \times 10^{-5} \text{ E/m}^2/\text{s}$ provided by warm white fluorescent lighting (Barghchi and Alderson, 1983b). Shoot proliferation after a few subcultures was approx. 12 shoots from each nodal bud explant in 6–8 weeks. Darkness during the first 7 days of incubation increases rooting in the presence of indolebutyric acid (IBA). More roots develop after shoots are transferred to auxin-free medium (Barghchi and Alderson, 1983a). Onay *et al.* (2004) successfully rejuvenated mature pistachio shoots by micrografting 'Siirt' shoot tips on *in vitro* seedlings; shoot tips of successful micrografts proliferated and plants were recovered.

There is a single report of axillary shoot initiation from explants of mature pistachio trees (Onay, 2000a). Mature pistachio cultivars have been extremely difficult to establish due to the high incidence of contamination (Barghchi, 1982; Bustamante-Garcia, 1984; Abousalim, 1990) and to oxidation of phenolic compounds (Barghchi and Alderson, 1989). Onay (1996) reported that small meristem tips (approx. 1 mm) from actively growing shoots of mature *P. vera* plants can be used to reduce the contamination problem. Multiple shoot production has been obtained from shoot tips cultured on semi-solid MS medium with B5 (Gamborg *et al.*, 1968) vitamins and 4.4 μM BA (Onay, 2000a), with a multiplication rate of 20 shoots for each explant for each 30-day cycle. Rooting of shoots has been induced on MS medium supplemented with 9.8 μM IBA. Rooted plantlets can survive in soil after a short acclimatization period.

4. Somatic Cell Genetics

Genetic manipulation of cell cultures has been demonstrated to be an efficient way to address breeding objectives (Wising *et al.*, 1998), and is essential for the future improvement of this crop.

Table 2.3.1. *In vitro* shoot tip/nodal culture studies of pistachio, *Pistacia vera* L.

Species and cultivars	Explant source	Medium (μ M)	Morphogenic response	References
<i>P. vera</i> L. rootstocks	tb/ab	MS + BA (18)	Multiple shoot proliferation Plantlet regeneration	Barghchi, 1982
<i>P. vera</i> L. rootstocks	tb/ab	MS + BA + NAA $\frac{1}{2}$ MS	Plantlet regeneration Callus formation	Alderson and Barghchi, 1982
<i>P. vera</i> L. rootstocks	st/ab	MS + kin (9.3–18) MS + kin (9.3–18) + NAA (1.4–1.5) + PhG Modified MS + IBA (12.3)	Single shoot growth Reduced multiple shoot Rooted plantlet	Barghchi and Alderson, 1983a,b
<i>P. vera</i> L.	ab	EM + BA (13.3) + GA ₃ (1.4) + IAA (0.006)	Shoot proliferation Plantlet regeneration	Bustamante-Garcia, 1984
<i>P. vera</i> L.	st		Shoot growth	Al Ramadhani, 1985
<i>P. vera</i> L. rootstocks	st/nb	MS + BA (18) Modified MS + IBA (12.3)	Multiple shoot proliferation Plantlet regeneration	Barghchi and Alderson, 1985
<i>P. vera</i> L. rootstocks	at/nb	MS + kin (9.3–18.6) MS + BA (18) Modified MS + IBA (12.3)	Single shoot growth Multiple shoot proliferation Rooted plantlet	Barghchi, 1986b
<i>P. vera</i> L. 'Kerman' and 'Peter's'	at/nb	MS + BA (1.3) + IBA (0.3) MS + 2,4-D + BA	Shoot growth Callus formation	Barghchi, 1986a
<i>P. vera</i> L.	st	MS + BA	Shoot growth and necrosis	Barghchi, 1986c
<i>P. vera</i>	st/ab	MS + BA	Shoot growth	Martinelli, 1988
<i>P. vera</i> L.	st		Plantlet regeneration Shoot growth	Gonzales and Frutos, 1990
<i>P. vera</i> L. 'Mateur'	st	MS + BA (17.8) MS + IBA (4.9–9.8)	Multiple shoot formation Rooting	Abousalim, 1990
<i>P. vera</i> L. 'Antep'	nb	MS + kin (4.7) + NAA (5.4)	Plantlet regeneration	Yücel <i>et al.</i> , 1991
<i>P. vera</i> L.	st/nb	MS + BA (0.4) + IBA (0.5) $\frac{1}{2}$ MS + IBA (7.4–12.3)	Multiple shoot formation Rooted plantlets	Yang and Lüdders, 1993
<i>P. vera</i> L. 'Mateur'	st	MS + BA (13.3)	Necrotic shoots	Abousalim and Mantell, 1994
<i>P. vera</i> L. 'Kerman' and 'Stewart'	st	MS + BA (4.4) + IBA (1.0) + MeJa (1.3– 4.4 μ M) NAA (31.7) + MeJa (4.5 μ M)	Shoot multiplication Rooted plantlets	Dolcet Sanjuan and Claveria, 1995
<i>P. vera</i> L.	st	DKW + BA + IBA DKW + IBA	Multiple shoot formation Rooted plantlets	Parfitt and Almehdhi, 1994
<i>P. vera</i> L. 'Antep'	nb	MS + BA (2.2–35.5)	Multiple shoot formation Plantlet regeneration	Onay, 2000a

ab, apical bud; ABA, abscisic acid; alf, aseptic leaf; at, apical tip; Au, auxin; BA, 6-benzylaminopurine; Cyt, cytokinin; 2,4-D, 2,4-dichlorophenoxy-acetic acid; DKW, Driver–Kuniyuki–Walnut medium; ER, Eriksson medium; GA₃, gibberellic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; if, immature fruits; ike, immature kernels; inf, inflorescence; ke, kernel; kin, kinetin; lf, leaf; MeJa, methyl jasmonate; MS, Murashige and Skoog medium; NAA, α -naphthalenacetic acid; nb, nodal bud; PEMS, proliferative embryogenic mass; PhG, phloroglucinol; st, shoot tip; tb, terminal buds; TDZ, thidiazuron.

Table 2.3.2. *In vitro* organogenesis and embryogenesis studies of pistachio, *Pistacia vera* L.

Species and Cultivars	Explant source	Medium (μ M)	Morphogenic response	References
<i>P. vera</i> L. Rootstocks	at/nb/lf	Mod MS + Cyt + Aux	Callus formation Organogenesis	Barghchi 1982; Barghchi and Alderson, 1983b
<i>P. vera</i> 'Lambertini'	at/nb/inf	MS + BA (1.3) + IBA (0.25) MS + 2,4-D + BA	Shoot growth Callus formation	Barghchi and Martinelli, 1984
<i>P. vera</i> L.	if	MS + 2,4-D (18) + NAA (10.7) + kin (9.3)	Callus formation	Zaheer <i>et al.</i> , 1989
<i>P. vera</i> L. 'Antep'	ke	MS + BA (8.9)	Somatic embryogenesis	Onay <i>et al.</i> , 1995
<i>P. vera</i> L. 'Antep'	st/nb	MS + NAA	Somatic embryogenesis	Taskin <i>et al.</i> , 1996
<i>P. vera</i> L. 'Antep'	PEMs	MS + BA (8.9)	Synthetic seeds Encapsulated PEMs	Onay <i>et al.</i> , 1996
<i>P. vera</i> L. 'Antep'	at/nb	MS + BA MS + BA or TDZ	Organogenesis Embryogenesis	Onay, 1996
<i>P. vera</i> L. 'Antep'	ke	Liquid MS + BA or ABA	Maturation Germination Embryo development	Onay <i>et al.</i> , 1997
<i>P. vera</i> L. 'Antep'	lf	MS + TDZ (4.5–9.1)	Somatic embryogenesis	Onay and Namli, 1998
<i>P. vera</i> L. 'Antep'	ike	MS + BA	Somatic embryogenesis Germination	Firat and Onay, 1999
<i>P. vera</i> L. 'Antep'	if/lf	MS + BA or TDZ	Somatic embryogenesis	Onay and Jeffree, 2000
<i>P. vera</i> L. 'Antep'	alf	MS + BA	Development of somatic embryos from single epidermal cells	Onay, 2000c
<i>P. vera</i> L. 'Antep'	ke	MS + BA	Somatic embryogenesis	Onay, 2000b
<i>P. vera</i> L. 'Antep'	at/nb	MS + BA or ABA	Somatic embryogenesis Maturation	Onay <i>et al.</i> , 2000

ab, apical bud; ABA, abscisic acid; alf, aseptic leaf; at, apical tip; Aux, auxin; BA, 6-benzylaminopurine; Cyt, cytokinin; 2,4-D, 2,4-dichlorophenoxy-acetic acid; DKW, Driver–Kuniyuki–Walnut medium; ER, Eriksson medium; GA₃, gibberellic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; if, immature fruits; ike, immature kernels; inf, inflorescence; ke, kernel; kin, kinetin; lf, leaf; MeJa, methyl jasmonate; MS, Murashige and Skoog medium; NAA, α -naphthalenacetic acid; nb, nodal bud; PEMs, proliferative embryogenic mass; PhG, phloroglucinol; st, shoot tip; tb, terminal buds; TDZ, thidiazuron.

4.1. Regeneration

4.1.1. Somatic embryogenesis

Somatic embryogenesis of pistachio was first achieved using immature kernels as explants (Onay *et al.*, 1995). Subsequently, somatic embryogenesis of several genotypes was reported using immature and mature zygotic embryos, cotyledons and juvenile leaf tissues

as explants (Taskin, 1995; Onay, 1996; 2000b,c; Taskin *et al.*, 1996; Onay *et al.*, 1997, 2000; Onay and Namli, 1998; Firat and Onay, 1999; Onay and Jeffree, 2000).

Induction. Kernels from immature fruits (12 weeks after pollination) have been used as explants. Following removal of the outer pericarp and shells, material is surface-sterilized, the testa is removed from each seed

and the kernels are washed with sterile distilled water before culturing in liquid MS medium, supplemented with B5 vitamins, 200 mg/l casein hydrolysate, 50 mg/l ascorbic acid, 8.9 μ M BA and 30 g/l sucrose. Cultures are incubated in continuous light at $25 \pm 2^\circ\text{C}$. Embryogenic cultures have also been induced from juvenile leaves. Onay and Jeffree (2000) reported induction of embryogenic cultures from juvenile leaves of pistachio on medium containing thidiazuron (TDZ). Embryogenic cultures consist of proembryonal masses (PEMs), which are evident after 14 days. The induction of somatic embryos from leaf explants of pistachio involves single epidermal or subepidermal cells (Onay, 2000c). Induction of embryogenic cultures from mature tissue explants has not been reported.

Maintenance. Embryogenic cultures consisting of PEMs can be maintained on induction medium supplemented with 4.4 μ M BA and on induction medium without growth regulators with subcultures at 12–14 day intervals. Cultures have survived for >2 years on plant growth regulator-free medium. When somatic embryos are transferred on to medium for development, abnormal embryos are recultured on induction medium in order to produce more PEMs.

Maturation. Proembryonal masses are transferred on to semi-solid MS medium containing 17.9 μ M BA and 60 g/l sucrose in order to initiate somatic embryo development. Approximately 49 somatic embryos develop from each 250 mg fresh weight (FW) of PEMs. A week after transfer of PEMs on to maturation medium, cotyledonary somatic embryos are evident.

Germination. Germination occurs on semi-solid or in liquid MS medium with B5 vitamins without phytohormones, and with 40 g/l sucrose, 500 mg/l casein hydrolysate and 50 mg/l ascorbic acid in continuous light. Onay *et al.* (1997) reported that liquid MS medium is optimal for recovery of plantlets; however, the rate of conversion is low. Onay *et al.* (2000) also observed that abscisic acid (ABA) is superior to BA for somatic embryo germination although BA is superior to ABA

for promoting subsequent development of plantlets. Somatic embryo-derived plantlets have been successfully transferred to soil mixture. Plantlets were covered with a beaker to maintain $90 \pm 5\%$ relative humidity (RH) for 4–5 weeks before transfer to a glasshouse (25°C day, 20°C night; 18 h photoperiod).

4.1.2. Organogenesis

Organogenesis has been reported from inflorescences, zygotic embryos and cotyledons (Barghchi and Alderson, 1983a,b, 1985; Barghchi and Martinelli, 1984; Bustamante-Garcia, 1984; Al Ramadhani, 1985; Barghchi, 1986a,b,c; Martinelli, 1988; Zaheer *et al.*, 1989; Abousalim, 1990; Gonzales and Frutos, 1990; Mederos and Carreno, 1991; Yücel *et al.*, 1991; Yang and Lüdders, 1993; Parfitt and Almehdhi, 1994; Dolcet Sanjuan and Claveria, 1995; Taskin, 1995; Onay, 1996, 2000a).

Modified MS medium is optimum for establishing caulogenic cultures from juvenile tissues (Barghchi and Alderson, 1989; Abousalim, 1990; Onay, 1996). Cytokinin is essential for shoot induction and multiplication (Barghchi and Alderson, 1983a,b; Parfitt and Almehdhi, 1994; Onay, 1996, 2000a). Shoots have been rooted on MS semi-solid medium containing auxin, but transfer to an auxin-free medium is necessary for vigorous root growth.

4.2. Genetic manipulation

4.2.1. Mutation induction and somaclonal variation

Genetic diversity in pistachio has been studied using morphological, physiological and biochemical data (Zohary, 1952; Lin *et al.*, 1984; Caruso *et al.*, 1988). To evaluate the genetic stability of pistachio plantlets obtained via organogenesis or somatic embryogenesis, the chromosome number of root tips has been counted (Onay 1996; Onay and Jeffree, 2000). Onay (1996) confirmed that somatic embryos derived from PEMs are $2n = 2x = 30$. Neither aneuploidy nor mixoploidy has been observed, although some differences in chromosome morphology were seen within a complement.

4.2.2. Genetic transformation

There have been no reports of genetic transformation of pistachio; however, the *in vitro* prerequisite, efficient somatic embryogenesis, is available.

4.3. Germplasm storage

PEMs and somatic embryos have been encapsulated in calcium alginate beads (Onay *et al.*, 1996) and stored for 60 days at 4°C. The encapsulated-stored PEMs proliferate after 2 months' storage and following two subcultures. The conversion frequency of encapsulated somatic embryos was 14%. Either approach can be used for short- and medium-term storage of pistachio genotypes and for the management of culture stocks during production.

5. Conclusions

The application of classical breeding to the pistachio tree has had only limited success due to the heterozygous nature of pistachio trees. The integration of genetic engineering into pistachio breeding programmes may overcome some of the limitations of conventional breeding. The establishment of sound protocols for regeneration from elite material is an essential prerequisite for the production of improved cultivars using modern techniques.

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3

Annonaceae

The family *Annonaceae* is within the order *Magnoliales*, class *Magnoliopsida*, which includes the most primitive angiosperms. The family includes 126 genera and approx. 1200 species, distributed for the most part in the tropical and subtropical areas of Africa, America, Asia, Australia and Europe (Watson and Dallwitz, 1992 onwards). There are several large genera, including *Annona* (150 spp.), *Guatteria* (265 spp.), *Duguetia* (100 spp.), *Uvaria* (100 spp.) and *Polyalthia* (100 spp.); some genera, e.g. *Annona*, *Anaxagorea* and *Xylopia*, are distributed almost worldwide. The taxonomy of the family is currently under revision (Maas *et al.*, 1994; Chatrou, 1998, 1999).

Most species in the *Annonaceae* are medium-sized, low-branched trees, although the family also includes small shrubs, tall canopy trees and lianas. The flowers are hermaphroditic and appear singly or in small clusters and exhibit both dichogamy and protogyny. Morphologically, the flowers possess three fleshy outer petals and three inner smaller petals. Fruits are usually nearly round, ovoid, conical and heart-shaped; the skin is covered by fingerprint-like markings or conical-rounded protuberances (areoles) (Morton, 1987). Only three genera, *Asimina*, *Rollinia* and *Annona*, produce edible fruits and only a few *Annona* species are grown commercially.

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3.1 *Annona* spp. Atemoya, Cherimoya, Soursop and Sugar Apple

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1. Introduction

1.1. Botany and history

By far the most important *Annona* species are *A. cherimola* Mill. (cherimoya), *A. muricata* L. (soursop), *A. squamosa* L. (sugar apple) and the hybrid *A. cherimola* × *A. squamosa* (atemoya) (Rasai *et al.*, 1995). Other *Annona* species, such as *A. diversifolia* (ilama), *A. reticulata* (custard apple), *A. glabra* (pond apple), *A. purpurea* (soncoya) and *A. scleroderma* (cawesh), represent potentially useful genetic diversity.

Archaeobotanical studies have dated *Annona* exploitation and cultivation in the Yau-tepec River region of Mexico to approximately 1000 BC (Ruiz-Sarabia and Moret-Alatorre, 1997). The first botanical references to the *Annona* genus appeared in the 16th century and described morphologically different species (Hernández, 1959). They have been dispersed by seed to many tropical and subtropical countries, and have become naturalized in many places. The origins of *A. cherimola*, *A. muricata* and *A. squamosa* are controversial and uncertain. It is generally accepted that they originated in different areas of Central and South America (Popenoe, 1921), and they have become naturalized in many tropical and subtropical areas through seed dispersal by humans. In contrast, the origin of atemoya is well docu-

mented, since the first hybrids were produced by Wester in Miami, Florida, USA, in 1908 and seedling trees were planted in 1910.

1.2. Importance

Species within the *Annonaceae* are a source of medicinal and pharmacological products, e.g. acetogenins (Cave *et al.*, 1993; McLaughlin, 1996; Gritsanapan, 1997; Ruiz-Sarabia and Moret-Alatorre, 1997), high-quality wood (Conafrut, 1974) and fruit. The major world producer of cherimoya is Spain, with approximately 3000 ha and 30,000 t fruit production per annum (Calatrava-Requena, 1992), followed by Chile and Peru. World cherimoya production is estimated to be approximately 50,000 t per annum. Smaller production occurs in Argentina, Brazil, Bolivia, Ecuador, Central America, Haiti, Jamaica, Mexico and Venezuela, as well as in Europe (Italy and Madeira), Africa (Algeria, Egypt, Eritrea, Libya and Somalia) and Asia (Sri Lanka, India and the Philippines).

Australia is the major producer of atemoya, with approximately 300 ha and 3000 t fruit production per annum (R.H. Broadley, Australia, 2001, personal communication), and there is small-scale production in Egypt, Florida (USA), India, Israel and South Africa.

There are no production data available for sugar apple and soursop. Sugar apple is widespread in the tropics, and soursop cultivation is restricted to the tropical lowlands of the Caribbean region, South and Central America, south-eastern China, Australia, Africa and South and South-east Asia.

1.3. Breeding and genetics

Atemoya, soursop, cherimoya and sugar apple have traditionally been propagated by grafting or budding commercial varieties on to seedling rootstocks. Propagation of *Annona* species by cuttings or air layering is difficult because of their low rooting potential. As seedlings are highly heterozygous, this produces heterogeneous plantations, with pronounced differences in productivity and vegetative characteristics, due to the variable performance of the seedling rootstocks. This has promoted the development of alternative propagation and breeding approaches for *Annona* fruit crops (Rasai *et al.*, 1995; Encina *et al.*, 1999a).

There has been little genetic improvement of *Annona* species. They are currently considered minor fruit crops due to the strict environmental requirements for tree plantings and the short postharvest life of their fruits. Horticultural and genetic studies have also been very limited. The chromosome numbers for *A. cherimola*, *A. squamosa*, *A. muricata* and *A. cherimola* × *A. squamosa* are $2n = 2x = 14$ (Nakasone and Paull, 1998).

Most *Annona* spp. are open-pollinated and fruit set is complicated by the short life of the pollen and the difficulty of the pollination mechanism (Hermoso-Gonzalez *et al.*, 1997). The market demands fruit with a regular shape; however, poor insect pollination, e.g. insufficient number of pollen grains, promotes the irregular thickening of the fruit carpels, resulting in asymmetric fruit shape. Hand pollination is necessary, thereby increasing the cost of production. Determining the kinetics of *in vitro* pollen germination (Rosell *et al.*, 1999) and the development of an easy and reliable test for pollen viability could improve fruit set of these species.

Different degrees of graft compatibility have been recorded among different *Annona* species (Vidal Hernandez, 1997). *A. muricata* (fibreless variety) and *A. squamosa* are graft-incompatible. *A. muricata* is graft-compatible with *A. montana* and *A. muricata* (fibreless variety) and to a lesser degree with *A. glabra* and *A. reticulata*. *A. cherimola*, *A. purpurea*, *A. muricata* and *A. spinosa* show low vegetative compatibility with one another. *A. cherimola* and *A. squamosa* are only partially graft-compatible, but cherimoya and *A. squamosa* are fully graft-compatible with atemoya (Nakasone and Paull, 1998).

1.3.1. Rootstocks

Major breeding objectives and accomplishments.

Dwarfing. There has been relatively little work on selection for dwarfing rootstocks. In Spain, several accessions (604–6, 'Piña' and 'Cholan' (SP78)) are currently under evaluation for reduced tree vigour. In Australia, George *et al.* (1999a,b) described semi-dwarf and dwarf genotypes among the progenies of the cherimoya 'Whaley'.

Root diseases. The soil fungi *Armillaria mellea* Vahl Kummer and *Rhizoctonia* spp. and the oomycete *Phytophthora* spp. cause damage to roots of most *Annona* spp. in water-saturated soils (Zarate-Reyes, 1995; Susham *et al.*, 1996; Weinert *et al.*, 1999; C.J. Lopez-Herrera, Spain, 2001, personal communication). Two rootstocks (*Rollinia* sp. and *Rollinia emarginata*) with increased root rot tolerance and graft compatibility with atemoya and cherimoya have been selected in Brazil (Bonaventure, 1999).

Soil stress. A Spanish cherimoya local selection 'Negrito' is under evaluation as an option for suboptimal environmental conditions, including heavy and poor soils.

1.3.2. Scions

Major breeding objectives.

Fruit quality. The major breeding objectives for atemoya and cherimoya include

improved fruit quality and better commercial cultivars. In Australia, the progeny of controlled pollinations are selected on the basis of the following parameters: (i) productivity, including precocity of bearing, percentage flower set, self-pollination, fruit set and yield; (ii) fruit quality, including a high flesh:seed ratio, average fruit size, skin characteristics (smooth, tolerance of bruising, attractive colour and lack of russetting), good flavour, sweetness and texture, pest and disease tolerance and round and symmetrical fruit shape; and (iii) postharvest characteristics, including extended storage life (> 10 days) and absence of discoloration.

Efforts to improve *A. muricata* and *A. squamosa* by conventional breeding have not been reported. There has been limited collection of genetic resources and some evaluation for red/pink skin and flesh and seedlessness of *A. squamosa* (Morton, 1987; Mahdeem, 1990). Some of the selections from these introductions could be useful for intra- or interspecific crosses in the future.

Control of ripening. *Annona* fruits are climacteric with a short shelf-life. Storage of fruits at low temperature is not very effective for prolonging shelf-life. In addition, half-ripened or ripe fruits are very fragile. The short harvest period and rapid fruit ripening have stimulated the introduction of cultivars having different maturity periods in order to even out market supply. Other possible methods that are being tried to extend storage life include manipulation of flowering and fruit set by controlled pruning (L. Soler-Marquessinis, Spain, 2000, personal communication) and the control of ripening by genetic transformation with ripening related genes in antisense (J. Botella, Australia, 2000, personal communication).

Fruit diseases. *Annona* fruits are susceptible to anthracnose (*Colletotrichum gloeosporioides* Penz.), and its incidence increases in warm and humid environments (Alvarez-Garcia, 1949; Dhingra *et al.*, 1980; Pennisi and Agosteo, 1994; Zarate-Reyes, 1995). To date, genotypes with resistance to anthracnose have not been reported.

Tree architecture. Elaborate models of plant management and pruning have been developed to increase planting density and to optimize harvesting. Development of an improved trellis training system and careful pruning and micropruning of young trees, increases efficiency of orchard management (Bonaventure, 1999).

Breeding accomplishments. Improvement of *Annona* spp. in Florida and the Philippines was reported by Wester (1913, 1915); however, there were few results from these early studies. Currently, there are two breeding programmes for atemoya, in Australia (George *et al.*, 1999b) and Florida, USA (Mahdeem, 1990), both of which are utilizing introductions of selected genotypes from Central and South America.

Fruit quality. A cherimoya breeding programme, which involves collection and evaluation of genotypes, has been under way in Spain since 1979, and includes 284 entries in the germplasm bank (J.M. Farre, Spain, 2001, personal communication). Studies on interspecific cross-pollination have been carried out (George *et al.*, 1997). Hybrid recovery studies involving polycrosses with *A. cherimola*, *A. squamosa*, *A. diversifolia*, *A. reticulata*, *A. scleroderma* and *A. cherimola* × *A. squamosa* have demonstrated sexual incompatibility between *A. scleroderma* and *A. reticulata*, resulting in non-viable hybrid seedling progeny. Partial sexual incompatibility has been recorded between atemoya 'Priestly' and *A. reticulata* 'Fairchild Purple', resulting in non-productive hybrids. In general, results from interspecific crosses show high variability in the F₁ progeny, showing segregation into parental types at variable ratios.

In Florida, USA, and Australia, interspecific crosses have been made between *A. cherimola* 'Spain' × *A. reticulata*, *A. cherimola* 'Mossman' × *A. diversifolia*, *A. cherimola* 'Libby' × *A. squamosa*, atemoya 'African Pride' × *A. cherimola* 'Spain', atemoya 'African Pride' × *A. reticulata*, atemoya 'African Pride' × *A. diversifolia*, atemoya 'African Pride' × *A. squamosa*, atemoya 'Hillary White' × *A. squamosa*, atemoya 'Priestly' × *A. reticulata* 'Fairchild Purple',

atemoya 'Gefner' $\times$ *A. reticulata* 'San Pablo' and *A. scleroderma* $\times$ *A. reticulata* (George *et al.*, 1997). Unfortunately, the F_1 progeny appear to be of little use because of poor fruit quality, except for some progenies in which *A. diversifolia* has been utilized as a parent. Some F_2 lines under evaluation are promising, having purple, red or pink skin. To date, a single advanced selection, 'Maroochy Gold', obtained by crossing atemoya 'Hillary White' and a red-skinned *A. squamosa*, seems to have commercial potential (George *et al.*, 1999a).

In Australia and Spain, F_1 selections from allelic crosses among different commercial cultivars of cherimoya 'White', 'Whaley', 'Booth', 'Fino de Jete', 'Sabor', 'Deliciosa', 'Bays', 'Ott' (Australia) and 'Cholan' (SP-78), 'Bonita', 'Fino de Jete', 'Pazicas' (Spain) are currently in progress. The selection criteria include fruit quality and productivity.

2. Molecular Genetics

2.1. Gene cloning

The first successful attempts at gene cloning have been made. Messenger RNA has been isolated from ripening fruits of atemoya and cherimoya. The gene for 1-aminocyclopropane 1-carboxylate (ACC) synthase has been cloned and sequenced from ripening fruit of atemoya and cherimoya (Encina *et al.*, 2001a,b). ACC synthase and other ripening-related genes are present at different degrees during fruit maturation, and this gene should be useful for developing transgenic atemoyas and cherimoyas with extended shelf-life by blocking ethylene emission. Atemoya and cherimoya ACC synthase sequences are available in the GenBank: AF401757 (Encina *et al.*, 2001a) and AF443280 (Encina *et al.*, 2001b). There is no information with respect to gene cloning of soursop or sugar apple.

2.2. Marker-assisted selection

2.2.1. Protein markers

There are relatively few reports involving molecular markers in *Annona* species.

Pascual *et al.* (1993) and Perfectti and Pascual (1998) used isozymes as genetic markers to characterize accessions of cherimoya and atemoya from the world germplasm bank of cherimoya (Spain). Thirteen enzyme systems (acid phosphatase (ACPH), alcohol dehydrogenase (ADH), diaphorase (DIA), glutamate oxalacetate transaminase (GOT), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), malic enzyme (ME), phosphoglucose isomerase (PGI), phosphoglucose mutase (PGM), 6-phosphogluconate dehydrogenase (6PGDH), shikimate dehydrogenase (SKDH), superoxide dismutase (SOD) and triose phosphate isomerase (TPI)), encoded by 23 loci, were studied. Fifteen loci displayed polymorphism and allowed the identification of most cultivars.

2.2.2. DNA markers

Ronning *et al.* (1995) applied random amplified polymorphic DNA (RAPD) analysis for fingerprinting genotypes of atemoya ('Ubrantzki', 'Malali' and 'Kaspi'), sugar apple ('Lessard') and cherimoya ('Fino de Jete' and 'Campas') and for study of the genetic variability among these selections. Using 15 Operon primers (A3, A14, A16, A17, A18, A20, B11, B18, C11, C19, D11, E3, E7, H20) (Operon Technologies, California), highly distinct, polymorphic patterns were generated. A segregating F_1 population of 'Fino de Jete' $\times$ 'Lessard' was also studied to determine parentage relationships.

Rahman *et al.* (1997) studied the genetic relationships among *A. cherimola*, *A. squamosa*, *A. reticulata*, *A. glabra*, *A. muricata*, *A. montana* and *A. cherimola* $\times$ *A. squamosa* by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis, using one set of sequence-tagged site (STS) primers. The results indicated that cultivated and wild species grouped separately; atemoya was located between its parents and *A. glabra* was placed in a separate cluster in the dendrogram.

Rahman *et al.* (1998) studied the genetic diversity of 19 cherimoya cultivars using amplified fragment length polymorphism (AFLP) analysis. A combination of *EcoRI*

primers (E-AGT) with *Mse*I adaptors (M-1 through M-8) was used. E-AGT/M-8 was the most efficient combination, yielding 21 polymorphic bands. The high number (264) of AFLP bands obtained, increasing by about eightfold the number of bands generated by RAPDs, suggests that AFLPs have greater utility for determining genetic diversity and parental relationships of cherimoya cultivars. Microsatellite markers have become the favoured method for fingerprinting most plant species (Gupta and Varshney, 2000). Their use with *Annona* spp. has great potential for species and genotype identification, for systematics and phylogenetic relationships and in marker-assisted selection to accelerate current breeding programmes.

3. Micropropagation

Regeneration of shoots from existing meristems of *Annona* spp. can be achieved after explant browning (Rasai *et al.*, 1994) and contamination (Tazzari *et al.*, 1990) are controlled, depending on genotype characteristics, e.g. dwarfing rootstocks. *Annona* spp. retain most of their morphogenic potential during the juvenile stage; however, rooting capacity disappears almost completely following phase change, although differences do occur among species and genotypes. Difficulty of rooting is typical of horticulturally interesting *Annona* selections, which are at the adult stage when selection has been made.

Nair *et al.* (1984b), working with atemoya (*A. cherimola* × *A. squamosa*), developed a protocol using nodal explants from juvenile trees. Multiple shoot initiation (six to seven axillary shoots per culture) was obtained using semi-solid Murashige and Skoog (1962) (MS) medium supplemented with 8.9 µM benzyladenine (BA); however, shoots were miniaturized and it was necessary to elongate them on a medium with low cytokinin content before rooting was feasible. Rooted plantlets were transplanted to soil and were acclimatized, with an 80% survival rate. Rasai *et al.* (1993) developed a system to stimulate partial autotrophy in

atemoya seedlings grown *in vitro*, using high irradiance, high relative humidity and low sucrose concentration in the culture medium.

Tazzari *et al.* (1990) worked with juvenile and mature nodal sections of *A. cherimola*, but were able to establish only juvenile explants *in vitro*. Encina *et al.* (1994) developed a protocol for micropropagation from nodal explants of juvenile *A. cherimola* 'Fino de Jete'. After sprouting and multiplication of axillary shoots on MS basal medium supplemented with either BA (0.7 µM) or zeatin (1.4 µM), a 95% rooting efficiency was achieved in three steps (Encina, 1992): (i) pretreatment of *in vitro* shoots for 7 days on semi-solid MS medium containing activated charcoal (0.1%); (ii) root induction on semi-solid MS medium supplemented with 500 µM indolebutyric acid (IBA), 58.4 mM sucrose and 200 mg/l citric acid for 10 days (7 days in darkness followed by 3 days of continuous light); and (iii) elongation of roots in half-strength semi-solid MS medium. Acclimatization is approx. 100% (Encina *et al.*, 2001a,b). Padilla (1997), using a modification of this protocol, succeeded in initiating shoot multiplication with adult explants from 18-year-old cherimoya 'Fino de Jete', while rooting and acclimatization were about 30% less efficient (I.M.G. Padilla and C.L. Encina, unpublished results).

Micropropagation is being adapted to other selected cherimoya cultivars and rootstocks. A new system for aseptic establishment of explants is under study and involves a modified micrografting protocol in cascade, which increases the sprouting and shoot development of all cultivars assayed. Although photoautotrophic conditions do not improve the efficiency of micropropagation (Encina *et al.*, 1999b), inoculation of micropropagated plantlets with arbuscular mycorrhizal fungi (*Glomus* spp.) improves vegetative growth and development of plantlets in the glasshouse and substantially reduces acclimatization time (Azcón-Aguilar *et al.*, 1994a,b, 1996).

Lemos and Blake (1996a) reported the micropropagation of *A. squamosa* from axillary shoots from 3-year-old trees. Following culture of explants on semi-solid woody plant medium (WPM) (Lloyd and McCown,

1981) containing 0.5 mg/l silver thiosulphate and 9 μ M BA, four shoot buds per explant elongated. Preconditioning of shoots for 2 weeks on WPM medium with 10 g/l activated charcoal was required before rooting on WPM containing 43 μ M naphthaleneacetic acid (NAA) or 39 μ M IBA, and rooting percentages improved if sucrose was replaced with 47% galactose. Following acclimatization, 80% of plantlets survived transfer to soil.

Lemos and Blake (1996b) developed a micropropagation protocol for juvenile and adult *A. muricata*. Axillary shoots on nodal explants were multiplied on WPM medium containing 0.5 μ M NAA and 8.9 μ M BA. Shoots were preconditioned on WPM with 3% activated charcoal and rooted in liquid WPM containing 21.5 μ M NAA and 1% galactose. Approximately 70% rooting occurred under these conditions and all rooted plantlets were acclimatized.

4. Somatic Cell Genetics

4.1. Regeneration

4.1.1. Organogenesis

Hypocotyl tissue exhibits high morphogenic competence in most *Annona* species, forming adventitious shoots and roots, and highly morphogenic callus.

Cherimoya.

Juvenile phase. Jordan (1988) inoculated *A. cherimola* 'Concha Lisa' hypocotyl sections, 0.3–1 cm long, into liquid MS medium supplemented with 2.2 μ M BA, 2.7 μ M NAA and 100 μ M polyvinylpyrrolidone (PVP) and in liquid Nitsch medium supplemented with 0.4 μ M BA and 0.4 μ M NAA. Adventitious buds developed without a callus phase in all of the explants. Hypocotyl segments, 2.5–3 cm long, rooted efficiently. Encina *et al.* (1999a), working with hypocotyls obtained from aseptically germinated seeds of cherimoya 'Fino de Jete', reported similar results.

Jordán *et al.* (1991), working with cherimoya zygotic embryos as primary explants,

also reported regeneration. Direct shoot formation from embryonic tissues and from embryo-derived calluses was obtained on Nitsch medium containing 0.5 μ M 2,4-dichlorophenoxy-acetic acid (2,4-D) and either 4.6 μ M zeatin or 4.4 μ M BA. For shoot regeneration, callus was transferred to Nitsch medium supplemented with 0.3 μ M indoleacetic acid (IAA) and 0.02% casein hydrolysate.

Adventitious shoot development from leaf and internodal explants of juvenile cherimoya seedlings ('Fino de Jete') has also been obtained (J.M. Cazorla and C.L. Encina, unpublished results). Bud clusters from juvenile explants developed and rooted easily. Organogenesis occurred directly from internodal explants. Lionakis and Nzuzi Gianze (2000) reported regeneration of shoot buds from single nodal segment explants from cherimoya seedlings on half-strength MS medium supplemented with 3.5% sucrose and 4.4–88.8 μ M BA and confirmed the adventitious origin of buds in anatomical studies.

Mature phase. Jordán *et al.* (1991) induced adventitious shoots from internodal sections of 3-year-old (mature phase) trees of 'Concha Lisa' on semi-solid Nitsch basal medium (Nitsch and Nitsch, 1969) supplemented with 2.7 μ M NAA, 8.9 μ M BA, 0.1% (w/v) PVP-360 and 0.02% casein hydrolysate. Antioxidants and sorbitol improved organogenesis. Adventitious shoot development from leaf and internodal explants of adult 'Fino de Jete' has also been obtained (J.M. Cazorla and C.L. Encina, unpublished results); shoot induction from adult phase explants required an elongation treatment to develop shoots. Little or no callus was implicated in organogenesis from internodal explants. Shoot regeneration from callus can be induced from mature leaves, but buds required an elongation treatment for shoot development.

Sugar apple. Lemos and Blake (1996a) induced multiple shoot buds from 1.5 mm hypocotyl segments of sugar apple on WPM containing 18 μ M BA. After pretreatment of shoots for 2 weeks on WPM with 10 g/l

activated charcoal, rooting and acclimatization frequencies of 47% and 80%, respectively, were achieved. Nair *et al.* (1984a) regenerated sugar apple adventitious buds and shoots from leaf explants. Their regeneration protocol included MS basal medium supplemented with 2.3 μM kinetin and 8.9 μM BA, and resulted in 15.6 shoot buds from each explant. Explant type was critical, since different shoot induction frequencies were obtained from various regions of the leaves. The most responsive area consists of the leaf base with petiole (15.6 shoots) and the least responsive area is the apical leaf region with midrib, from which there was no shoot bud induction. Supplementing standard regeneration medium with different auxins promoted callus growth and fewer shoot buds. The efficiency of rooting of regenerated shoots and the recovery of complete plantlets after acclimatization was very low (10%).

Soursop

Juvenile phase. High-frequency adventitious shoot induction occurs from hypocotyl segments (1 cm length) from aseptically germinated seeds of *A. muricata* on semi-solid MS medium supplemented with 8.9 μM BA and 0.5 μM NAA (Bejoy and Hariharan, 1992). Rooting (88%) is induced on semi-solid MS medium with 9.8 μM IBA and plants have been acclimatized (60%). Lemos and Blake (1996b) induced adventitious shoots from hypocotyl explants on semi-solid WPM medium containing 2.7 μM NAA and 8.9 μM BA. Following shoot preconditioning treatment on WPM with 3% activated charcoal for 2 weeks, 100% rooting was observed after 15 days incubation in darkness in liquid WPM medium with 21.5 μM NAA and 1% galactose. Eighty per cent of the plantlets were successfully acclimatized.

Mature phase. Adventitious shoot regeneration was reported from 67% of internodal explants of mature phase soursop on semi-solid Nitsch medium supplemented with 8.9 μM BA, 2.7 μM NAA, 0.1% PVP, 0.02% casein hydrolysate and 2% sorbitol (Lemos and Baker, 1998).

Atemoya

Juvenile phase. Rasai *et al.* (1994) induced adventitious shoot formation (12.6 per explant) from hypocotyl segments of atemoya 'African Pride' on semi-solid MS medium containing 8.9 μM BA, 2.3 μM kinetin, 0.4 μM biotin and 0.4 μM calcium pantothenate.

Mature phase. Rasai *et al.* (1994) also reported induction of adventitious shoots (19 shoots from each explant) from nodal explants of mature tree origin ('African Pride') on the same medium. Approximately 40% rooting of shoots from mature phase trees was obtained following a two-step rooting method: (i) root induction in liquid MS medium containing 250–500 μM IBA for 3 days in darkness, followed by 13 days constant illumination; and (ii) root elongation on semi-solid half-strength MS medium with 0.25% activated charcoal. A 70% survival rate was obtained after acclimatization in the glasshouse.

4.1.2. Haploid recovery

Nair *et al.* (1983) described haploid induction from cultured anthers of *A. squamosa*. Dissected anthers from 1–1.5 cm flower buds, containing mid-uninucleate and uninucleate pollen grains, formed callus on Nitsch medium supplemented with 28.6 μM IAA. Callus formation from anthers required a preliminary incubation period of 7 days in darkness. Regeneration of single shoots from callus occurred on Nitsch medium supplemented with 8.9 μM BA and 5.4 μM NAA. Multiple shoot development occurred on Nitsch medium supplemented with 8.9 μM BA and 0.6 μM IAA. The haploid number of regenerated plantlets ($n = x = 7$) was verified.

4.1.3. Triploid recovery

A ruminant type of endosperm is characteristic of the *Annonaceae*. Attempts to regenerate triploid ($3n = 21$) plants from endosperm tissue have had limited success. Shoots or roots, but not complete plantlets, have been regenerated from mature endosperm of *A.*

squamosa (Nair *et al.*, 1986). Endosperm was dissected from sterilized mature seeds and explanted 2–4 days after germination. Seeds were pretreated for 1 h with 0.3 mM gibberellic acid (GA_3) and incubated on White's basal medium (White, 1963) supplemented with 2.9 μ M GA_3 , 5.4 μ M NAA, 0.9 μ M BA and 0.5 μ M kinetin. Triploid shoots were induced from endosperm callus on Nitsch medium supplemented with 8.9 μ M BA and 2.7 μ M NAA, and triploid root induction occurred on medium supplemented with 28.6 μ M IAA.

4.1.4. Protoplast isolation and culture

Protoplasts have been isolated from two different explant types: hyperhydric leaves and etiolated hypocotyl tissue, both obtained from *in vitro*-germinated seedlings of *A. cherimola* 'Fino de Jete'. The stock plantlets were preconditioned for 24 h at 10°C (C.L. Encina, unpublished results). The leaf epidermis was removed and scarified, and hypocotyls were macerated. Both explants were preplasmolyzed for 2 h at 25°C in the dark on an orbital shaker (40 rpm) prior to enzymatic digestion. For preplasmolysis, CPW salt formulation (Frearson *et al.*, 1973) supplemented with 0.6 M mannitol and 3 mM methyl ethane sulphonate (MES) (pH 5.8) was used. The enzyme solution consisted of CPW salts supplemented with 2000 mg/l inositol, vitamins of Kao and Michayluk (1975), 0.6 M mannitol, 3 mM MES and filter-sterilized 1.2% cellulase Onozuka RS (Yakult Honsha Co., Tokyo, Japan), 0.2% Macerozyme R10 (Yakult Honsha Co.) and 0.25% pectolyase Y-23 (Sigma). After overnight digestion in darkness under stationary conditions plus 2 h of gentle shaking (40 rpm) on an orbital shaker, protoplasts were separated from the digestion mixture by filtration through 40–60 μ m nylon mesh. Protoplasts were concentrated in a pellet after 5 min centrifugation at 100 g in 50 ml sterile centrifuge tubes, and carefully resuspended in 1 ml CPW modified by increasing $CaCl_2 \cdot 2H_2O$ to 25 g/l, and the protoplast suspension was diluted to 50 ml with this formulation. The mixture was centrifuged twice for 5 min, the supernatant was discarded, and the pellet was resuspended and homogenized in

fresh washing medium. Protoplast yields from leaf and hypocotyl tissue were 2×10^6 and 1×10^6 per gram, respectively.

Collected protoplasts were cultured at a density of 1.5×10^5 cells/ml in B5 liquid medium (Gamborg *et al.*, 1968), supplemented with 0.6 M mannitol, 0.09 M sucrose, 5 μ M 2,4-D, 3 μ M BA, 1 μ M zeatin, 2 μ M NAA and 1% dimethyl sulfoxide (DMSO). After 5 days' incubation in stationary dark conditions at 25°C, the first cell divisions occurred and the microcolonies were subcultured to fresh medium without 2,4-D, with reduced cytokinin concentration and gradually decreasing the mannitol concentration. Microcallus appeared in 6 weeks. Regeneration from protoplasts derived from leaf callus has not been observed; however, a low level of regeneration, approximately 0.5%, was obtained from protoplasts derived from hypocotyls, and half of the shoots were rooted. Efficiency of this protocol must be increased for it to have utility. This is the first report of protoplast isolation and culture of *Annona* spp.

4.2. Genetic manipulation

4.2.1. Genetic transformation

There has been very little work on genetic transformation of *Annona* spp., due to the current limitations of *in vitro* regeneration. Preliminary studies of *Agrobacterium tumefaciens*-mediated genetic transformation of atemoya and cherimoya have been attempted (J.R. Botella and C.L. Encina, Australia, 2001, personal communication).

Breeding objectives. Genetic engineering of *Annona* spp. has focused on control of fruit ripening in order to extend the shelf-life and fruit quality (seedlessness). Different strategies that have been adopted to achieve these goals include: (i) control of the expression of genes related to growth and development of seeds; and (ii) blocking the biosynthesis of ethylene by transformation with ACC synthase genes in antisense. In the future, improvement of orchard management, e.g. the development of dwarfing rootstocks by blocking GA_3 biosynthesis, could possibly be addressed.

Table 3.1.1. Current status of *in vitro* studies involving *Annona* species.

Species	Explant	Phase	Basal medium and addenda (mg/l)	Growth regulators (μ M)	Shoot growth	Rooting	Goal path	Plant recovery	References
<i>Annona cherimoya</i>	Internode	J	N + 200 CH + 1000 PVP	8.9 BA + 2.3 KN	67%	N.D.	AO	N.D.	Jordán <i>et al.</i> (1991)
	Nodal section	J	MS→ MS + 200 citric acid	0.7 BA or 1.4 Z→ 490 IBA→ 0 IBA	90%	—	MPP	—	Encina <i>et al.</i> (1994)
		A	MS→	2.3 Z→	40%	—	MPP	< 70%	Padilla (1997)
		J	MS + 200 citric acid	490 IBA→ 0 IBA	—	50%	MPP	60%	Jordán <i>et al.</i> (1991)
		J	N	4.4 BA + 0.5 NAA	40%	N.D.	MPP	N.D.	Lionakis and Nzuzi (2000)
		J	$\frac{1}{2}$ MS + 3.5% sucrose	4.4–88.7 BA	N.D.	N.D.	AO	N.D.	
<i>Annona muricata</i> sour sop	Leaf + petiole	J	MS + 200 CH	8.9 BA + 2.7 NAA	100%	N.D.	AO	N.D.	Jordán <i>et al.</i> (1988)
		J	N + 200 CH + 1000 PVP	8.9 BA + 2.7 NAA	60%	N.D.	AO	N.D.	Bridg (1993)
	Hypocotyl	J	MS + 1000 PVP→ MS + 1000 PVP	8.9 BA + 2.7 NAA→ 54 NAA	100%	—	AO	N.D.	Jordán <i>et al.</i> (1988)
		J	CPW/KM-8p→ B5 + 1% DMSO	5 2,4-D + 3.1 BA + 0.9 Z + 2.2 NAA	0.5%	N.D.	AO	N.D.	Encina (unpublished)
	Zygotic embryo	J	N→ N + 200 CH	4.4 BA or 4.6 Z + 0.5 2,4-D→ 0.29 IAA	30.8%	N.D.	AO	N.D.	Jordán <i>et al.</i> (1991)
		J	CPW/KM-8P→ B5 + 1% DMSO	5.0 2,4-D + 3.1 BA + 0.9 Z + 2.2 NAA	0%	0%	PROT	—	Encina (unpublished)
	Leaf	J							
	Pollen	J	BK1 + 50,000 sucrose	None	*50.5%	—	G	—	Rosell <i>et al.</i> (1999)
	Seed	J	DW	8.7 GA ₃	82.4%	82.4%	G, MG MPP	100%	Padilla (1997)
		A	N + 20,000 sorbitol	8.9 BA + 2.7 NAA	67%	N.D.	AO	N.D.	Lemos and Baker (1998)
<i>Annona muricata</i> sour sop	Nodal section	A	WPM→ WPM + 10,000 galactose	8.9 BA + 0.5 NAA→ 21.5 NAA	100%	— 70%	MPP MPP	— 100%	Lemos and Blake (1996b)

soursop (continued)	Hypocotyl	J	N + 200 CH + 1000 PVP	8.9 BA + 2.7 NAA	60%	N.D.	AO	N.D.	Bridg (1993)
	MS→	J	MS→	8.9 BA + 0.5 NAA→	100%	—	AO	—	Bejoy and Hariharan (1992)
	MS		MS	9.8 IBA	—	88%	AO	60%	
	WPM→	J	WPM→	8.9 BA + 2.7 NAA→	100%	—	AO	—	Lemos and Blake (1996b)
	WPM + 10,000 galactose			21.5 NAA	—	100%	AO	100%	
	DW	J	DW	None	N.D.	N.D.	G	N.D.	Bejoy and Hariharan (1992)
	DW	J	DW	None	N.D.	N.D.	G	N.D.	Lemos and Blake (1996b)
<i>Annona squamosa</i>	Nodal section	J	WPM + 0.5 ST→	8.9 BA→	N.D.	—	MPP	—	Lemos and Blake (1996a)
			WPM + 10,000 galactose	38.8 IBA	—	47%	MPP	80%	
sugar apple	Leaf + petiole	J	MS→	2.3 KN + 2.2 BA or 2.31 KN + 8.9 BA→	100%	—	AO	—	Nair et al. (1984a)
			$\frac{1}{2}$ AA + 2.5 AC	98 IBA	—	10%	AO	10%	
	Hypocotyl	J	WPM + 0.5 ST→ WPM + 10,000 galactose	8.9 BA→ 38.8 IBA	100%	—	AO	—	Lemos and Blake (1996a)
					—	47%	AO	80%	
	Anther	J	N	8.9 BA + 0.5 NAA	10%	N.D.	AO	N.D.	Nair et al. (1983)
	Endosperm	J	N	8.9 BA + 2.7 NAA	25%	5%	AO	N.D.	Nair et al. (1986)
	Seed	J	DW→ WhM	289 GA ₃ → 0 GA ₃	N.D.	N.D.	G	N.D.	Nair et al. (1986)
		J	DW	None	N.D.	N.D.	G	N.D.	Lemos and Blake (1996a)
<i>A. cherimola</i> × <i>A. squamosa</i>	Nodal section	A	MS + 0.1 biotin + 0.1 Ca-pantothenate + 1000 NH ₄ NO ₃ → MS→ liq. $\frac{1}{2}$ MS + 2500 AC	8.9 BA + 2.3 KN→ 245 IBA	N.D. —	—	AO	—	Rasai et al. (1994)
					—	41.6%	AO	70%	

Continued

Table 3.1.1.1. Continued.

Species	Explant	Phase	Basal medium and addenda (mg/l)	Growth regulators (mg/l)	Shoot growth	Rooting	Goal path	Plant recovery	References
atemoya	Hypocotyl	J	MS + 0.1 biotin + 0.1 ca-pantothenate	8.9 BA + 2.3 KN	100%	41%	AO	70%	Rasai et al. (1994)
		J	MS + 0.1 biotin + 0.1 Ca-pantothenate + 0.75% sucrose	8.9 BA + 2.3 KN	100%	N.D.	MPP	N.D.	Rasai et al. (1993)
	Seed	J	MS + 20,000 sucrose	8.7 GA ₃	N.D.	–	G	N.D.	Rasai et al. (1994)

A, adult; AA, Anderson medium (Anderson, 1980); AC, activated charcoal; AO, adventitious organogenesis; B5, B5 Gamborg medium (Gamborg et al. 1968); BAP, benzylaminopurine; BK1, modified Brewbaker and Kwack medium (Brewbaker and Kwack, 1963); CH, casein hydrolysate; CPW, CPW salts (Frearson et al., 1973); 2,4-D, 2,4-dichlorophenoxy-acetic acid; DMSO, dimethyl sulphoxide; DW, distilled water; G, germination; GA₃, gibberellic acid; IAA, indoleacetic acid; IBA, indole-3-butyric acid; J, juvenile; KM-8p, Kao and Michayluk medium (Kao and Michayluk, 1975); KN, kinetin; MG, micrografting; MPP, micropropagation; MS, Murashige and Skoog medium; N, Nitsch medium (Nitsch and Nitsch, 1969); NAA, naphthalene acetic acid; N.D., no data; PROT, protoplast; PVP, polyvinyl-pyrrolidone; ST, silver thiosulphate; WhM, White medium (White, 1963); WPM, woody plant medium (Lloyd and McCown, 1981); Z, zeatin.

5. Conclusions

Annona spp. are minor fruit crops; however, there is increased interest in such exotic fruits worldwide (Table 3.1.1). In the future, development of efficient methods for modifying ploidy of these species will result in the selection of triploid (seedlessness) genotypes and of dihaploid genotypes with valuable horticultural traits. Improved methods for regeneration of plants from somatic

tissues, together with genetic transformation, will facilitate genetic manipulation and improvement.

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4

Arecaceae

The subtropical to tropical monocotyledonous family *Arecaceae* (*Palmae*) belongs to the order *Spadiciflores*, suborder *Palmales*. There are at least 200 genera and at least 3000 species in the family of palms, including woody shrubs, vines or trees (Watson and Dallwitz, 1992 onwards), many of which are important sources of food, e.g. peach palm or pejiabaye *Bactris gassipaes* Kunth., coconut palm *Cocos nucifera* L., areca nut *Areca catechu* L., date palm *Phoenix dactylifera* L., fibre, e.g. rattan

palm *Calamus rotang* L., etc., and consumable oil, e.g. oil palm *Elaeis guineensis*. The fruit is usually a drupe. Within the *Arecaceae*, there are 19 tribes; *Bactris* spp., *Elaeis* spp. and *Cocos* spp. are in the *Cocoinae* and *Phoenix* spp. are in the *Phoeniceae*. The fruit of many species is edible, and the heart or shoot apex of many palms, but particularly *B. gassipaes*, is consumed in salads and as a vegetable. Many species are also important as landscape plants in the tropics and subtropics.

Reference

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4.1 *Cocos nucifera* Coconut

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1. Introduction

1.1. Botany and history

The coconut palm (*Cocos nucifera* L.) is a relatively slow growing woody perennial species. It is the only species in the genus *Cocos*. All forms known to date are diploid ($2n = 2x = 32$). No closely related species with even partial interfertility has been reported (Bourdeix *et al.*, 2001). The lifespan of a coconut palm can be > 60 years under favourable ecological conditions. Coconuts can grow to a height of approx. 25 m (Ohler, 1999).

Optimum growing conditions for coconut are in the lowland humid tropics at altitudes < 1000 m near coastal areas in sandy, well-drained soils (Persley, 1992); however, coconuts are adaptable to other soil types including coral atolls and soils with moderate salinity (Batugal, 1999). Coconuts are also commonly cultivated several hundred kilometres inland, e.g. surrounding Lakes Victoria, Tanganyika and Malawi in Africa (Lombard, 2001). Coconuts cannot tolerate temperatures < 0°C and ideal growing temperatures range between 24 and 30°C (Woodroof, 1979; Persley, 1992).

Coconuts do not form a tap root, but develop a fasciculated root system, consisting of adventitious roots at the base of the stem, which typically grow laterally to 2–3 m

length and 30–120 cm deep and continuously generate adventitious roots (Reynolds, 1988; Persley, 1992). Nutrients and water are absorbed by the rootlets.

The coconut palm ‘trunk’ is a stem with no true bark, no branches and no cambium. Secondary growth (increased stem diameter) is by secondary enlargement meristem located below the shoot meristem. Growth depends on age, ecotype and edaphic conditions, but is generally between 30 and 100 cm per annum. The stem is surmounted by a crown of approx. 30 compound leaves, which protect the terminal vegetative bud and whose destruction causes the death of the palm. An adult coconut has virtually as many unopened (20–30) as opened leaves. Leaves are produced continuously at approx. 1 month intervals.

The coconut palm is a monoic species. Flowering may begin between 3 and 10 years after planting. Each leaf bears an inflorescence primordium in its axil. The coconut inflorescence is a spadix, which develops within a double sheath referred to as a spathe. When mature, the spadix breaks through the spathe and 30–35 spikelets emerge, each bearing a large number of male flowers (200–300) with one or two female flowers at the base of each spikelet. Flowers are sessile and follow the trinary organization of monocotyledons (Menon and

Pandalai, 1958). Male flowers have three short sepals, three petals, six stamens and one rudimentary pistil. Female flowers are approx. 3 cm in diameter, and are enveloped by small scaly bracteoles enclosing three sepals and three petals, which overlap each other and surround the spherical pistil. The ovary is tricarpaceous and each carpel has a single ovule. After fertilization, a single ovule develops and the two others abort or degenerate. The inflorescence can be either self- or cross-pollinated (Bourdeix *et al.*, 2001). Pollination is by wind or insects.

The appearance of the fruit (size, shape and colour) varies according to the ecotype (Bourdeix *et al.*, 2001). The coconut is a drupe, whose development requires approx. 1 year. Only 25 to 40% of the female flowers develop into mature nuts and a tree produces < 100 fruits per annum. After fertilization, the husk and shell increase in size and the cavity of the embryo sac enlarges considerably (Menon and Pandalai, 1958). The cavity is filled with a liquid endosperm. After 6 months, the solid endosperm develops as a thin and gelatinous layer against the inner wall of the nut cavity (Ohler, 1999). After 8 months and towards the later stages of ripening, the endosperm becomes hard and white and is surrounded by a hard, brown testa (Ohler, 1984). The immature endosperm is composed of 95% water and < 1% oil, and 50% water and 30–40% oil at maturity (Ohler, 1984). When ripe, the nut generally falls. The seed, which is one of the largest in the plant kingdom, is characterized by lack of dormancy and the time necessary for development from embryo to plantlets (Blake, 1990; Verdeil, 1993).

Four months are generally required for the first leaf to emerge from the husk. A characteristic of coconut zygotic embryos is the substantial development of the haustorium (distal part of the cotyledon) within the nut cavity during germination (Menon and Pandalai, 1958). This organ invades the nut cavity and establishes intimate contact with the endosperm. It enables the hydrolysis of the endosperm and the mobilization of nutrients required for embryo germination. Lipase, protease and saccharase activity have even been detected (Bertrand, 1994).

Histological studies have demonstrated digitations in the epidermal layer in contact with the nutrient reserves, and the existence of vascular bundles converging towards the embryonic axis. This villosity displays numerous structural similarities to stomach villi in the digestive system of animals (Verdeil and Hoche, 2002).

Fossil nuts > 15 million years old and very similar to present-day coconuts have been discovered in New Zealand and India (Sauer, 1967, cited by Harries, 1978; De Taffin, 1998); however, the exact geographic origin of this species is uncertain. In all probability, the coconut tree was first cultivated either in India or in South-east Asia. The coconut has attained its highest development in terms of variability and number of local names in South-east Asia.

1.2. Importance

The coconut palm has been referred to as the 'tree of life', because of its importance as a subsistence crop in most tropical areas of the world. It is grown on > 11 million ha, 94% of which are in Asia and the South Pacific (Blake, 1990). World production of coconut has been estimated to be 52,940,408 t (FAOSTAT, 2004). The leading producers are Indonesia and the Philippines (> 13,000,000 t), India (9,500,000 t), Brazil (2,833,910 t), Sri Lanka (1,850,000 t), Thailand (1,400,000 t), Papua New Guinea (570,000 t), Vietnam (920,000 t) and Mexico (959,000 t). Many coconut-producing countries are small islands in the South Pacific and Indian Oceans and the Caribbean region (Daviron, 1995), where coconut can be grown in harsh environments, such as atolls, and can tolerate swampy and water-deficient areas and poor soils. Coconut is an important attribute of the rural economy (Punchihewa, 1999), and is cultivated by many farmers on small landholdings (< 4 ha) often in association with other crops (root crops, vegetables, cacao, etc.) (Barrant, 1978; Reynolds, 1988; Freud and Daviron, 1994). Only 10% of the planted areas constitute commercial plantations. Coconut palm is cultivated mainly for copra (dried endosperm) production, from

which oil is extracted and provides income for smallholders in the tropics and subtropics.

The coconut has been a primary source of food, drink and shelter for millions of people from the earliest days of humankind (Batugal, 1999; Punchihewa, 1999). Coconut farmers are deeply attached to the various products (Punchihewa, 1999), and have contributed to its adaptation to a wide range of environmental conditions. Although significant achievements have been made with respect to the release of high copra-yielding hybrids (Bourdeix *et al.*, 2001), this progress has yet to reach most coconut producers.

The coconut is mainly a subsistence crop, e.g. 70% of the production is consumed locally in Asia. Every part of the plant can be used. Oil from the fresh nuts is used for food preparation in many countries of Asia and the Pacific. The kernel can be oven- or sun-dried to a moisture content of 6% (copra), and can be conserved for months before oil extraction. Coconut water is a very refreshing drink. Endosperm of mature nuts is grated and used in pastries. The woody stem is used as a building material and in joinery. The leaves can serve for local handicrafts and as roofing material. The processed sap provides sugar, syrup and vinegar. The fibres from the husk surrounding the nut can be used to manufacture esparto-type goods. More ecofriendly than rock wool, these fibres can also be used as a substrate for growing plants (Bourdeix *et al.*, 2001).

Plantations were developed throughout the tropics by the end of the 19th century to satisfy the need for coconut oil for industrial uses (Daviron, 1995), including the extraction of glycerine, a component of dynamite. Until the mid-20th century, coconut was the main oil source in the world market. Coconut oil is extracted from the dried endosperm (copra) and, together with oil palm kernel oil, is the only source of short-chain fatty acids (from eight to 14 carbon atoms), and a rich source of lauric acid (~48%) (Persley, 1992). It is used in soap manufacture and in the cosmetic industry (Blake, 1990; Verdeil *et al.*, 1996a). The melting point of coconut oil is 24–27°C and hydrogenation is not required to inhibit rancidity because of its stability; coconut oil is

therefore widely used in food products (margarine, confectionery, etc.) (Ohler, 1984). With only 4% of the world oil production, coconut ranks seventh among oil-bearing crops. In the competitive international world oil market, the coconut palm is gradually being replaced by other oil-seed plants such as soya and oil palm (Freud and Daviron, 1994). The coconut palm is therefore reverting to a multipurpose crop, especially for its fruit. Several reasons can explain this gradual decline: (i) low productivity due to old age of coconut plantations (two-thirds of the individuals are > 60 years old) and insufficient replanting; (ii) use of unimproved material and marginal culture practices; (iii) several pests and diseases, e.g. lethal yellowing (LY) and Cadang-Cadang; (iv) production in areas often subjected to natural calamities, e.g. typhoons or volcanic eruptions; and (v) low prices for coconut oil despite its high quality and lower production (Freud and Daviron, 1994). In addition, rapeseed oil, which has been genetically modified to produce oil (Laurical®), with a higher content of lauric acid (37%), has had a significant impact on production. Despite these difficulties and stagnant production for 20 years, coconut oil is still important, and there continues to be demand for lauric oil for the soap industry (Freud and Daviron, 1994). With the assistance of the World Bank, the Philippines has started a replanting programme using improved hybrids, and LY was recently declared a national priority for research in Mexico (Aldaba, 1995; INIFAP, 1998). The CGIAR has even recognized coconut as the oil crop most in need of international research.

1.3. Breeding and genetics

1.3.1. Plant characteristics

Propagation is entirely by seed. Allogamy causes a high degree of variability. The breeding cycle is very long (12 to 16 years), with a low number of seeds produced (100 to 200 seeds/tree per annum) and a large recalcitrant seed that makes exchange and conservation of germplasm extremely difficult. These morphological and biological charac-

teristics impose serious constraints on breeding. There are three groups of coconut palms – Tall (*C. nucifera typica*), Dwarf (*C. nucifera nana*) and hybrids between the two. Tall palms represent the more common type and account for > 95% of coconut production because of their general superiority in copra production (Woodroof, 1979; Persley, 1992). Dwarfs are distinguished mainly by slower growth. They generally produce lower quality copra than Tall and for this reason are often not used for large-scale plantings (Woodroof, 1979). Dwarfs exhibit other features, e.g. preferential autogamy, reduction in organ size, early maturity and rapid fruit production. Because of these last two characters, Dwarfs are very important in breeding programmes (Bourdeix *et al.*, 2001).

1.3.2. Breeding objectives

The diversity of coconut uses ensures that there is no single ideotype. Breeding objectives are particularly complex, and include a tradeoff between food, cultural habits and processing requirements. The highest priority is increased production of copra per hectare (Bourdeix *et al.*, 2001). Other important objectives include precocity, adaptation to certain edaphoclimatic conditions (drought, cold, pH) and resistance to diseases. Several pathogens (see Table 4.1.3), including fungi (*Phytophthora* spp.), trypanosomes (heart rot), nematodes (red ring), viruses (coconut foliar decay (CFDV)), viroids (coconut cadang cadang (CCCVd)) and phytoplasma (LY) cause heavy losses. The genetic improvement of the coconut relies on exploitation of the variability within the species. Coconut breeding began in India in 1916 (Harries, 1978), although major progress was not obtained until the 1960s. Currently, 20 centres throughout the tropics are involved in coconut breeding.

Hybrids can include: Dwarf × Tall, Tall × Tall or Dwarf × Dwarf (Harries, 1991). According to Ohler (1984), breeders and growers prefer the Dwarf × Tall type because of early maturity, ease of production and seed whose quality can be readily controlled. Nevertheless, other hybrid types can also provide certain advantages depending on the

cultivation system and use. The breeding programme of the Centre de Coopération Internationale en Recherche Agronomique pour le Développement – Département Cultures Pérennes (CIRAD-CP) uses reciprocal recurring selection as a starting point. The method involves exploiting ecotype combining ability and basing phenotypic choices on heritable characters (Gascon and de Nucé de Lamothe, 1978) and has been described in detail by de Nucé de Lamothe (1970) and Gascon and de Nucé de Lamothe (1976). Genetic improvement involving hybridization between ecotypes has resulted in a doubling of the outputs within 20 years. The best hybrids can increase profits by 20 to 30% within a generation.

Genetic gain has been assisted by the development of reliable hybrid seed production techniques using assisted pollination (Wuidart and Rognon, 1981). Hybrids are reproduced on a large scale, e.g. 1 ha of seed-bearing trees can produce c. 15,000 seeds per annum by assisted pollination (de Nucé de Lamothe and Wuidart, 1992). This method is complex, costly and time consuming (de Nucé de Lamothe and Wuidart, 1992), requiring emasculation of female parents, conditioning and conservation of pollen from male parents and manual or assisted pollination (Wuidart and Rognon, 1981). The cost of a selected seednut can be as much as US\$2–4, which is too expensive for smallholders (Verdeil *et al.*, 1998a).

According to Baudouin (1999), the efficiency of breeding can be improved as follows: (i) combining genetically distant genotypes to increase heterosis; (ii) increasing selectable diversity in breeding populations; (iii) using molecular marker and quantitative trait loci (QTLs) to increase selection efficiency using marker-assisted selection (MAS); and (iv) using *in vitro* propagation for rapid dissemination of genetic gain (Verdeil *et al.*, 1995, 1998a).

2. Molecular Genetics

The application of MAS in coconut breeding is urgently needed because desired characters are expressed only after several years of

growth. The use of molecular markers offers certain advantages for identifying cultivars and for determining taxonomic relationships. The studied traits directly reflect variation that occurs within the genome, they are neutral and their expression is independent of the environment (Lebrun and Baudouin, 2002). Their use should increase the efficiency and efficacy of coconut genetic improvement, especially for germplasm management, genotype identification and MAS of important traits. In many species, molecular markers are being used to create genetic linkage maps in order to identify markers linked to specific traits that can form the basis for MAS. Construction of genetic maps would have great benefit for coconut.

2.1. Markers

Initial studies on genetic diversity characterization involved isozymes or polyphenol markers (Carpio, 1982; Canto-Canché *et al.*, 1983; Jay *et al.*, 1989; Fernando and Gajanayake, 1997; Cardeña *et al.*, 1998). The characterization of genetic diversity in coconut germplasm at the DNA level (Ashburner, 1999) has largely replaced these strategies. Various DNA markers have been used to measure coconut genetic diversity: inverse sequence-tagged repeat (ISTR) (Rohde *et al.*, 1995; Duran *et al.*, 1997); randomly amplified polymorphic DNA (RAPD) (Ashburner *et al.*, 1997; Duran *et al.*, 1997; Rodriguez *et al.*, 1997; Wadt *et al.*, 1999); restriction fragment length polymorphism (RFLP) (Lebrun *et al.*, 1998, 1999); amplified fragment length polymorphism (AFLP) (Perera *et al.*, 1998); simple sequence repeat (SSR) (Karp, 1999; Perera *et al.*, 1999; Rivera *et al.*, 1999; Teulat *et al.*, 2000). Two main coconut groups have been identified: Indian and Pacific Ocean. Analysis of DNA polymorphisms has indicated that the Tall and Dwarf types show different degrees of polymorphisms with more polymorphism in Tall types. Using microsatellites, a kit for identifying coconut cultivars is under development in CIRAD and should allow the large-scale application of molecular fingerprinting of coconut (Lebrun and Baudouin, 2002).

2.2. Linkage mapping and QTL analysis

In coconut, the availability of F_1 mapping populations from controlled crosses involving heterozygous parents has allowed linkage mapping of identified polymorphisms as well as the search for QTLs. An initial linkage analysis of the East African Tall (EAT) and Laguna Tall (LAGT) coconut types based entirely on ISTR markers was described by Rohde *et al.* (1999). This work was extended using AFLPs, ISTRs, RAPDs and inter-sample sequence repeats (ISSRs), and allowed the construction of a linkage map of the two parents of the cross involving Malayan Yellow Dwarf (MYD) $\times$ LAGT, resulting in 382 identified markers and 16 linkage groups generated for each parent and the identification of QTLs associated with early flowering and yield (Herrán *et al.*, 2000). In addition, QTLs for other traits, including leaf production and girth height, were identified for the same mapping population (Ritter *et al.*, 2000). AFLP and SSR markers have been used to construct a linkage map for a coconut type from the Solomon Islands, the Rennell Island Tall (RIT), which is used in various breeding programmes and as a male parent for commercial hybrids in the Pacific (Lebrun *et al.*, 2001). QTL analysis allowed the identification of loci linked to number of bunches and the number of nuts.

The identification of different QTLs provides the first opportunity for MAS in coconut. The most efficient use of MAS would be to produce parental lines for F_1 hybrid production and to search for LY-resistant hybrids (Cardeña *et al.*, 1999). According to Ashburner (1999), there is still a basic lack of knowledge of the genetics of the species. The large stature, long generation time and low multiplication rate will always hamper breeding. Molecular markers can minimize but not eliminate these problems.

3. Somatic Cell Genetics

3.1. Regeneration

Due to the time required in order to develop improved selections, micropropagation is

essential for distribution of selections that emerge from breeding programmes (Verdeil *et al.*, 1998a). Vegetative multiplication of elite selections is necessary for producing homogeneous planting material and thereby improving plantation productivity. Moreover, *de novo* regeneration of coconut is essential for genetic transformation; however, coconut palm is considered to be one of the most recalcitrant species for *in vitro* culture (Georges and Sherrington, 1984; Hoher *et al.*, 1999).

3.1.1. Somatic embryogenesis

Somatic embryogenesis involving different explant types has been attempted, including apical meristems (Hagedorn, 1990), young roots of mature palms (Justin, 1978), stems and leaves (Pannetier and Buffard-Morel, 1982; Gupta *et al.*, 1984; Raju *et al.*, 1984), zygotic embryos (Bhala-Sarin *et al.*, 1986; Karunaratne and Periyapperuma, 1989; Ueda *et al.*, 1993), inflorescences (Eeuwens, 1978; Branton and Blake, 1984; Sugimura and Salvana, 1989; Verdeil *et al.*, 1989, 1993) and plumules from mature embryos (Hornung, 1995, 1997; Chan *et al.*, 1998).

Induction. The primary explants for embryogenic culture must contain meristematic tissue, which proliferates in the presence of an auxin. Immature leaves and inflorescences are the most useful explants, as the phenotype of the mother tree is already known. Inflorescences are generally preferred because of a simplified protocol and an inflorescence sampling protocol which does not result in death of the tree (Rillo, 1989). Plumules (embryo meristem with the first primordium) have been utilized (Hornung, 1995, 1997), and this pathway can be exploited as a model for developing protocols using other explants and to multiply the progeny from selected parents (Saenz *et al.*, 1999).

Somatic embryogenesis generally occurs indirectly by directive induction; however, there is a single report of direct embryogenesis from leaf explants (Raju *et al.*, 1984), which is unusual since vascular tissue normally produces root primordia (Blake, 1989).

Embryogenic cultures are induced from explanted tissues collected from adult coconut palms on various culture media. At the Institut de Recherche pour le Développement (IRD)/CIRAD, the Eeuwens Y3 mineral solution (Eeuwens, 1976) is used with Morel and Wetmore's vitamins (1951), 40 g/l sucrose, 7.5 g/l agar, 2 to 2.5 g/l activated charcoal and 99.55 to 271.5 μ M 2,4-dichlorophenoxyacetic acid (2,4-D), due to the variable sensitivity between palms to auxin at pH 4.5–5.8 (Verdeil *et al.*, 1999). Murashige and Skoog medium (1962) (MS) with the addition of sucrose, activated charcoal and auxin is also employed. The cultures are usually incubated in the dark at 27°C (Buffard-Morel *et al.*, 1992; Verdeil *et al.*, 1994). Activated charcoal is necessary to control browning, which is a major constraint of coconut *in vitro* culture (Blake and Eeuwens, 1980, 1981; Pannetier and Buffard-Morel, 1986; Tisserat, 1990). The effect of activated charcoal appears to be due to reversible adsorption of the auxin and its slow and gradual release (Brackpool *et al.*, 1986; Ebert and Taylor, 1990; Ebert *et al.*, 1993; Verdeil *et al.*, 1999). The auxin 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) has also been used for induction of nodular calluses from inflorescence explants (Buffard-Morel *et al.*, 1988; Verdeil and Buffard-Morel, 1995). The histology of callus has been studied (Buffard-Morel *et al.*, 1992; Verdeil *et al.*, 1992).

Callus grown on media with a gradually reduced auxin level (Blake, 1990) or with an increase followed by a reduction of auxin (Verdeil *et al.*, 1994) will eventually produce nodular structures (Fig. 4.1.1) that subsequently develop into proembryos (Fig. 4.1.2). Absciscic acid (ABA) appears to affect the formation of coconut proembryos (Samosir *et al.*, 1999b; Fernando and Gamage, 2000). Histological studies of embryogenic cultures indicate that there are two developmental pathways. A multicellular pathway occurs on medium with 2 g/l activated charcoal and 181–362 μ M 2,4-D (Buffard-Morel *et al.*, 1992; Verdeil *et al.*, 1992, 1994), but has also been observed on medium containing ABA (Fernando *et al.*, 2003). Embryogenic cultures typically consist of meristematic and proembryonic structures. Initially, cells in the

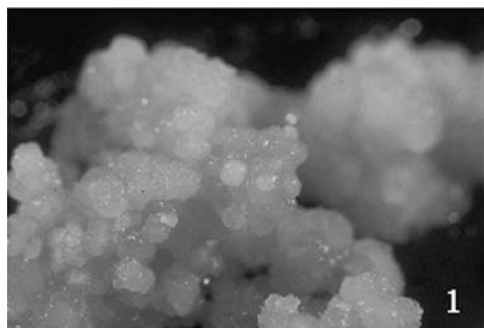


Fig. 4.1.1. Coconut embryogenic culture.

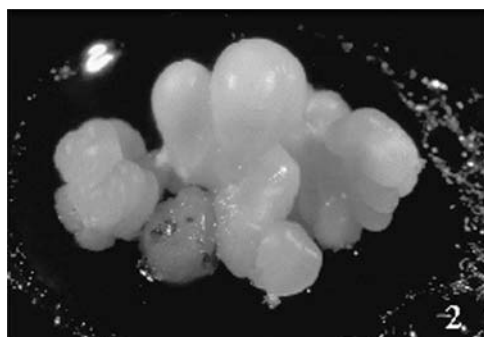


Fig. 4.1.2. Globular stage coconut somatic embryos.

cambium-like zones proliferate, and actively dividing cells give rise to meristematic nodules that develop a protoderm or epidermis. Proembryos develop from proembryonic cells in the periphery; however, if the auxin concentration is too low, anomalous structures, e.g. haustorium only, a root pole, foliar-type somatic embryos, etc., can develop (Branton and Blake, 1983; Brackpool *et al.*, 1986).

Another pathway occurs in the presence of 2–3 g/l charcoal and 362–543 μM 2,4-D, whereby individual embryos develop from single embryogenic cells (Schwendiman *et al.*, 1988; Verdeil *et al.*, 1994). In that case, typical proembryos develop according to the description by Haccius and Phillip (1979). The embryogenic cells have dense cytoplasm, a high nucleo-cytoplasmic ratio, a single and voluminous nucleolus and many starch and protein reserves. They become separated from the culture as a result of cell wall thickening (Lu and Vasil, 1985; Williams

and Maheswaran, 1986; Schwendiman *et al.*, 1988). There are deep invaginations of the nuclear envelope, proliferation of dictyosomes and emission of Golgi vesicles, which is directly related to increased cell wall thickness (Verdeil *et al.*, 2001). Seven to 14 days after explanting, callose deposition blocks the plasmodesmata, resulting in physiological isolation. Acquisition of embryogenic competence was linked to the appearance of an outer layer of pectic material (mainly non-methyl-esterified) that entirely coats the embryogenic cells (21 days after explanting) (Verdeil *et al.*, 2001). Specific nutrient requirements have been observed (Dussert *et al.*, 1995a,b; Magnaval *et al.*, 1995, 1997). Tyrosine phosphorylated proteins and tyrosine kinase activity increase under induction conditions (Islas-Flores *et al.*, 2000). A similar observation has been made during coconut zygotic embryo development (Islas-Flores *et al.*, 1998, 2000).

Maintenance. Embryogenic cultures, irrespective of origin, are slow growing and nodular, and proliferation occurs from the peripheral region (Buffard-Morel *et al.*, 1992). Embryogenic cultures are maintained on a proliferation medium based on MS macro- and Nitsch (1969) micro-elements, Morel and Wetmore (1951) vitamins, 40 g/l sucrose, 2 g/l activated charcoal and 7.5 g/l agar. This medium is supplemented with 271.5–362 μM 2,4-D. Cultures are maintained in darkness and subcultured every 2 months.

Maturation. Somatic embryo development is asynchronous and occurs from < 10% of cultures. Regeneration of complete somatic embryos requires lower 2,4-D concentrations (181–271.5 μM) (Fig. 4.1.3). Thidiazuron (TDZ) or 2-isopentenyladenine (2iP) has been utilized effectively to stimulate development (Verdeil *et al.*, 1996b). Somatic embryos are maintained in the dark and subcultured every 2 months until shoot emission. Differentiation of the shoot meristem of the somatic embryo is cytokinin-dependent (Verdeil *et al.*, 1994) and has been corroborated by the increase in isopentenyl forms of cytokinin during early somatic embryo development (Hochoer *et al.*, 1998a).

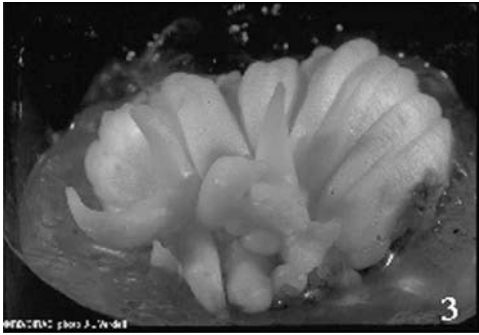


Fig. 4.1.3. Somatic embryos during the maturation phase.

Germination. Germination of the somatic embryos occurs on maturation medium containing benzyladenine (BA). Gibberellic acid (GA_3) can promote somatic embryo germination in the presence of BA (Fig. 4.1.4). Cultures are transferred to the light after the development of two to four leaves. Root induction can be promoted by naphthaleneacetic acid (NAA). Maturation and acclimatization of plantlets are major bottlenecks for regeneration by somatic embryo-

genesis. Foliar development is very slow and is sometimes associated with leaf chlorosis. The physiological status of *in vitro* shoots has been studied using *in vitro*-germinated zygotic embryos as a model. Different photosynthetic parameters have been studied (Triques *et al.*, 1997a,b): (i) chlorophyll fluorescence to determine photosynthetic efficiency; (ii) activities of phosphoenolpyruvate carboxylase (PEPC) and ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) were determined and the PEPC:RubisCO ratio was used as an indicator of autotrophism; (iii) net photosynthesis rate was estimated through CO_2 exchange measurements; and (iv) chloroplast ultrastructure. A lower rate of net photosynthesis was recorded for *in vitro*-grown plantlets compared with acclimatized palms, possibly due to lower RubisCO activity together with lower chlorophyll content compared to acclimatized plants (Triques *et al.*, 1998). Santamaria *et al.* (1999) demonstrated that sucrose lowered RubisCO activity, while slightly increasing the activity of PEPC. Since PEPC/RubisCO is a measure of plant photoautotrophy (Desjardins, 1995), these

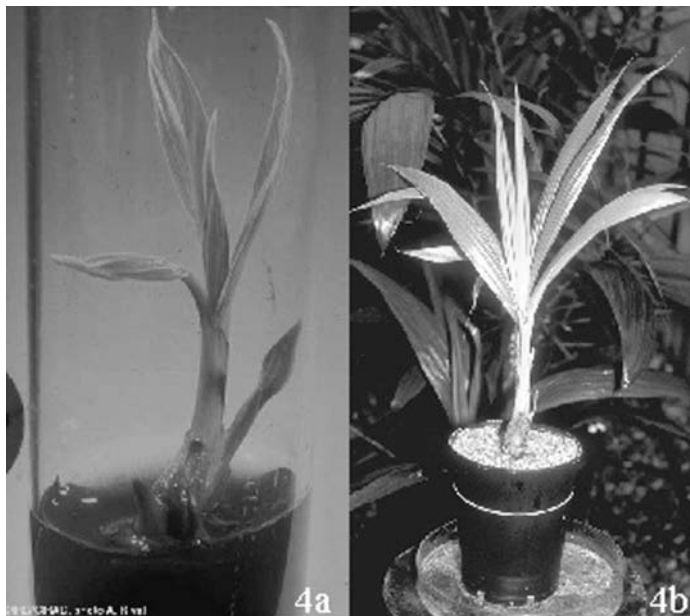


Fig. 4.1.4. Coconut somatic plantlets from the test tube (a) to the greenhouse (b).

results suggest that sucrose inhibits the development of photoautotrophy *in vitro*. They suggested that sucrose might be important in early stages of somatic embryo development; however, continuous growth in sucrose-rich medium in later stages could affect photoautotrophism and also plant performance *ex vitro*.

In vitro-grown plants (derived from zygotic embryos) have reduced capacity to control water loss compared to field-grown plants, due to altered stomatal functioning. Ventilation of the culture containers resulted in an increased capacity of *in vitro*-grown plants to control water loss (Talavera *et al.*, 2001). These results have implications for *in vitro* hardening and acclimatization.

3.1.2. Haploids

Haploidy is of great interest considering the allogamy of numerous coconut varieties and hybrids (Than-Tuyen and De Guzman, 1983). Monfort (1985) and Thanh-Tuyen (1985) reported promising results but no regeneration, and they were unable to recover complete embryos. More recently Griffis and Litz (1997) obtained proembryos from cultured anthers, anther filaments and unfertilized ovary cultures on medium containing diethylstilboestrol; however, no further development was reported.

3.1.3. Protoplast isolation and culture

Haibou and Koor (1981) described the isolation of protoplasts from immature inflorescence rachillae and microcallus

regeneration from some of them. Unfortunately, a low rate of division was observed in coconut protoplast cultures and no regeneration was reported.

3.2. Conservation

Coconut seeds have no dormancy, causing problems in transporting and storing germplasm (Assy-Bah *et al.*, 1987; Engelmann and Dussert, 2000). Coconut genetic resources are maintained in field collections (Verdeil *et al.*, 1996a) in five countries: Côte d'Ivoire, Indonesia, India, Papua New Guinea and Vanuatu. The Côte d'Ivoire collection is the most important in terms of genotypic diversity, with 24,962 accessions including 53 ecotypes (36 Tall types represented by 20,600 palms and 17 Dwarf types represented by 4200 palms) and 12 inter-ecotype hybrids (Bourdeix *et al.*, 1998; N'Cho *et al.*, 1998). *Ex situ* conservation is costly, and collections are subject to diseases and climatic adversity. The Coconut Genetic Resources Network (COGENT) was created in 1992 with the support of the International Plant Genetic Resources Institute (IPGRI) to bring together 35 producing countries in order to maintain and protect coconut genetic resources (Baudouin *et al.*, 2000; Table 4.1.1). The highest priority is to duplicate field collections *in vitro* as pollen and embryos (Ramanatha Rao and Batugal, 1998) and to facilitate international exchange of germplasm. Short- and medium-term storage *in vitro* is essential for conservation of germplasm that is free of known diseases,

Table 4.1.1. Countries with an international coconut genetic resources database (CGRD). Coconut germplasm collections with passport and characterization data: a French-funded project. Number of accessions per country. (Adapted from Batugal, 1997, 1999; Baudouin *et al.*, 2000.)

Latin America/ Caribbean				South-east Asia					
Africa	na	na	na	South Asia	na	na	na	South Pacific	na
Benin	4	Brazil	16	Bangladesh	4	Indonesia	156	Fiji	11
Côte d'Ivoire	99	Jamaica	60	India	212	Malaysia	92	Papua New Guinea	57
Tanzania	72	Mexico	20	Pakistan	32	Philippines	224	Vanuatu	66
				Sri Lanka	78	Thailand	52	Western Samoa	9
						Vietnam	31	Solomon Islands	21
Total per region	175		96		326		555		164

na, number of accessions.

and represents the safest method for international exchange of material (Withers and Williams, 1985). It is also a prerequisite for cryogenic storage.

Routine techniques for collecting zygotic embryos have been developed, including field collection, disinfecting and embryo culture (Assy-Bah *et al.*, 1987; Rillo, 1995; Ashburner *et al.*, 1996; Samosir *et al.*, 1999a; Karun, 2001; N'Nan *et al.*, 2002a). Excised embryos can be stored in KCl for up to 14 days before *in vitro* culture (Assy-Bah *et al.*, 1989). Coconut embryo culture was initially developed in the Philippines for embryo rescue of 'Makapuno', a highly valued Philippine mutant genotype (De Guzman and Del Rosario, 1964; Del Rosario, 1998). Karunaratne *et al.* (1991) used coconut embryo culture to measure drought tolerance in Sri Lanka, and were able to screen a large number of genotypes in a short time (2 years). Rillo (1985) used embryo rescue to screen for disease tolerance.

Different protocols for embryo culture have been described (Del Rosario and De Guzman, 1976; Karunaratne *et al.*, 1985; Assy-Bah, 1986; Sossou *et al.*, 1987; Assy-Bah *et al.*, 1989; Rillo and Paloma, 1991; Karun *et al.*, 1993; Ashburner *et al.*, 1996; Rillo, 1999). Low germination and survival rates of plants *ex vitro* indicate that the protocol requires improvement. An international programme coordinated by COGENT has begun to focus on improving *in vitro* culture and acclimatization protocols (Batugal and Engelmann, 1998).

Zygotic embryos can be stored *in vitro* for medium-term periods (6 to 12 months) without loss of germination (Assy-Bah and Engelmann, 1993; M'kumbo, 1995). Development can be suppressed by high levels of sucrose and activated charcoal (Assy-Bah, 1992; Verdeil *et al.*, 1998b). Increased osmolarity and reduction of nutrient concentration can also impede development (Damasco, 2002). None the less, long-term conservation by cryopreservation is essential to reduce the loss of important genetic resources.

Early attempts to cryopreserve coconut embryos by Bajaj (1984) and Chin *et al.* (1989) were not very successful. Assy-Bah and Engelmann (1992a,b) demonstrated that

mature coconut embryos could be cryopreserved after 4 h desiccation in a laminar air flow followed by immersion for 11–20 h in a cryoprotectant consisting of 600 g/l glucose and 15% glycerol (Assy-Bah and Engelmann, 1992b). Four coconut varieties (hybrid PB121, Indian Tall, Cameroon Red Dwarf and Rennell Island Tall) were successfully cryopreserved with a germination rate of 10–93%, depending on ecotype. These results were validated with West African Tall (WAT) and MYD (N'Nan, 1997), and later with ten more ecotypes (N'Nan *et al.*, 2003).

Plumules have been cryopreserved by encapsulation/dehydration (N'Nan, 1999; Malaurie and Borges, 2001; Malaurie *et al.*, 2002). Plumules were excised and encapsulated in alginate beads, and exposed to different sucrose concentrations and dehydration periods, resulting in 40–80% survival after cryopreservation. Up to 70% of plumules of some ecotypes germinate normally following cryopreservation (Malaurie and Borges, 2001; N'Nan *et al.*, 2002b; Fig. 4.1.5). Hornung *et al.* (2001) cryopreserved plumules, and attempted to induce embryogenic cultures according to the protocol of Chan *et al.* (1998). Other cryopreservation techniques, e.g. encapsulation, osmoprotection, dehydration and encapsulation, osmoprotection and vitrification (Sakai *et al.*, 2000), have been applied to plumular tissues, and shoot development has been reported (Malaurie *et al.*, 2003).

Hybridization and improved nut production are facilitated by assisted pollination (Wuidart and Rognon, 1981; de Núc de



Fig. 4.1.5. Somatic embryo development from dehydrated, encapsulated and frozen plumule.

Lamothe and Wuidart, 1992). According to Towill (1985), palms have long-lived pollen; however, for long-term breeding programmes, extended storage of pollen is essential (Towill and Walters, 2000). Coconut pollen storage was reported by Whitehead (1965) using freeze-drying. Pollen desiccation to 4–5% moisture content over silica gel, followed by storage *in vacuo* in a freezer, does not cause loss of viability for > 6 months (Rognon and de Nuccé de Lamothe, 1978). Cryopreservation of pollen is also feasible (Frison *et al.*, 1993; Engelmann, 1999), and recommendations for collecting, conditioning and cryogenic storage of pollen have been reported (Frison *et al.*, 1993).

Technical guidelines for the safe movement of coconut germplasm have been established (Frison *et al.*, 1993; Diekmann, 1997; Baudouin, 1998; Table 4.1.2). Indexing techniques for screening germplasm for known diseases is critical, e.g. CFDV, which causes foliar decay in Vanuatu, CCCVd in the Philippines and LY, a phytoplasma-associated disease, which has caused great devastation in the Caribbean region and more recently in Ghana (Harrison *et al.*, 1999; Rodriguez, 1999). All of these diseases (Table 4.1.3) should be prevented from being transferred outside their current area of distribution (Frison *et al.*, 1993; Diekmann, 1997, 1999; Hanold and Randles, 1997; Dollet, 1999; Hodgson and Randles, 1999; Howard and Harrison, 1999; Jones *et al.*,

1999; Nair *et al.*, 1999). A list of treatments has been proposed for controlling the spread of these diseases in the technical guidelines for the safe movement of coconut germplasm (Table 4.1.4). There are no therapies for eliminating coconut virus, viroid and phytoplasma diseases of coconut. Reverse transcription polymerase chain reaction (RT-PCR) has demonstrated the presence of LY phytoplasma in embryonic tissue, including the plumule (Cordova *et al.*, 2003). Exchange of coconut germplasm by means of zygotic embryos corresponds to the basic Food and Agriculture Organization (FAO)/International Board for Plant Genetic Resources (IBPGR) guidelines for moving coconut germplasm (Diekmann, 1997, 1999; Ramanatha Rao and Batugal, 1998); however, existing indexing protocols do not provide adequate security. *In vitro* collections of coconut germplasm are located in six coconut-producing countries and two European countries (Table 4.1.5).

The establishment of the multi-site International Coconut Genebank (ICG), hosted by India, Indonesia, Papua New Guinea and Côte d’Ivoire for their respective regions, will have the responsibility to conserve and share a maximum of 200 important accessions from South and South-east Asia, the Pacific region and Africa and Indian Ocean islands, respectively (Table 4.1.6). The accessions maintained in ICG will include: (i) the principal varieties; (ii)

Table 4.1.2. Summary of FAO/IBPGR Technical Guidelines for the Safe Movement of Coconut Germplasm. General recommendation: to move embryo culture or pollen, not nuts. (Adapted from Harrison *et al.*, 1995; Diekmann, 1997; Ramanatha Rao and Batugal, 1998; Dollet *et al.*, 2001a,b.)

Pathogen	Specific recommendation
CFDV	Indexing or exclusion of germplasm from Vanuatu
CCCVd	Indexing or exclusion of germplasm from the Philippines
CtiVd	Indexing or exclusion of germplasm from Guam
Viroid-like sequence	Indexing or exclusion of germplasm that is moved from countries where these sequences are known to occur to countries where they have not yet been reported. Recommendation under revision
LY, phytoplasma	Transmission through seed, embryo culture or pollen not reported, but suspicion still exists
Kerala wilt, phytoplasma	
Tatipaka disease, phytoplasma	A nursery disease which does not occur on adult trees
Blast, phytoplasma	

CtiVd, coconut tinangaja viroid.

Table 4.1.3. Causal agent, vector, final disease evolution, geographical distribution of the coconut diseases, and techniques available for indexing (adapted from Frison *et al.*, 1993; Hanold and Randles, 1997; Diekmann, 1999; Dollet, 1999; Hodgson and Randles, 1999; Howard and Harrison, 1999; Jones *et al.*, 1999; Nair *et al.*, 1999; Dollet *et al.*, 2001).

Type of disease	Disease name	Cause	Vector	Final disease evolution	Geographical distribution	Indexing: conventional techniques	Indexing: molecular approach
Viral	Foliar decay	Coconut foliar decay virus (CFDV); icosahedral virus	<i>Myndus taffini</i> (Cixiidae) planthopper	In susceptible coconut palms, the crown dies within 6 months to 2 years	Vanuatu, and suspected in other areas	–	Dot-blot hybridization and complementary labelled DNA probe
Viroid	Coconut cadang-cadang	Coconut cadang-cadang viroid (CCCVd); circular single-stranded RNA in a rod-like structure	Field and seed transmission are observed and pollen suspected. Mechanism of transmission remains unknown	8 to 16 years elapse between first symptoms and death of the palm. Some palms die soon, those that continue to develop never flower	Occurs in certain parts of the Philippines	PAGE	MHA. Hybridization analysis with radioactive RNA probes (Northern blotting); Rt-PCR
	Coconut tinangaja	Coconut tinangaja viroid (CtiVd); single-stranded circular RNA	Means of natural transmission unknown	Diseased palms decline and die in similar manner to cadang-cadang	Guam	PAGE	Hybridization analysis with radioactive probe
Viroid-like – sequences	–	Viroid-like sequence similar to but not identical to CCCVd	Means of natural transmission unknown	–	South Asia to French Polynesia	–	Northern blotting technique with a complementary RNA probe specific to CCCVd
Mollicute	Blast	Mycoplasma-like organism (MLO)	<i>Recilia mica</i> Kramer (Jassidae)	–	Africa, and South America and Indonesia for similar symptoms	–	–
	Lethal yellowing (LY)	Phytoplasma	<i>Myndus crudus</i> (Cixiidae) planthopper; suspicion over different phloem-feeding insects for LY in Africa	The whole of the crown eventually rots and falls off within 3–6 months of the appearance of the first symptoms. Complete destruction of plantation in Mexico	Africa, Central America and Caribbean	Light or electron microscopy with fluorescent staining (DAPI)	Amplification by PCR of the 16–23S rRNA region of phytoplasma
	Root wilt or Kerala wilt	Mycoplasma-like organism (MLO)	<i>Stephanisus typica</i> , <i>Proustia moesta</i> (putative vector)	Symptoms appeared only on 30-month-old palms. The disease is not lethal, but significantly reduces production	India (parts of Kerala and Tamil Nadu states)	Light microscopy with fluorescent staining (DAPI)	PCR
	Taipaca disease	Mycoplasma-like organism (MLO)	Unknown	The disease is not lethal, but significantly reduces production	India (East and West Godavari, Srikakulam and Nellore in Andhra Pradesh)	Light microscopy with fluorescent staining (DAPI)	PCR
	Heartrot disease	Trypanosomatid	Pentatomid bugs from the genus <i>Lincaus</i>		Surinam, Salvador de Bahia Province, north Honduras, Trinidad, Costa Rica	40 x 10 phase-contrast light microscope	None

DAPI, 4'-6-diamidino-2-phenylindole; MHA, Mueller-Hinton agar; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription polymerase chain reaction.

Table 4.1.4. Therapy available against the different coconut diseases (adapted from Frison *et al.*, 1993; Diekmann, 1997).

Disease name	Cause	Therapy
Foliar decay	CFDV	None
Coconut cadang-cadang	CCCVd	None
Coconut tinangaja	CtiVd	None available
	Viroid-like sequence	None
Blast	Phytoplasma, MLO	None
Lethal yellowing (LY)	Phytoplasma, MLO	Tetracycline, but no elimination of the phytoplasma from palms
Root wilt or Kerala wilt	Phytoplasma, MLO	Tetracycline, but no elimination of the phytoplasma from palms
Tatipaca disease	Phytoplasma, MLO	Tetracycline, but no elimination of the phytoplasma from palms

Table 4.1.5. COGENT member countries concerned in international exchange of coconut (*Cocos nucifera* L.) germplasm, and expected COGENT member countries (adapted from Batugal, 1997, 1999).

Africa	Latin America/ Caribbean	South Asia	South-east Asia	South Pacific
Côte d'Ivoire	Brazil	Bangladesh	China	Cook Islands
Ghana	Costa Rica	India *	Indonesia *	Fiji
Kenya	Cuba	Pakistan	Malaysia	Kiribati
Mozambique	Guyana	Sri Lanka *	Myanmar	Papua New Guinea *
Nigeria	Haiti		Philippines *	Solomon Islands
Seychelles	Jamaica		Thailand	Tonga
Tanzania	Mexico *		Vietnam	Vanuatu
	Trinidad-Tobago			Western Samoa
Possible future members				
Comoro	Colombia			Marshall Islands
Madagascar	Dominican Republic			Tuvalu
	Ecuador			
	El Salvador			
	Guatemala			
	Panama			
	Venezuela			

In bold, regional coconut genebank, also called International Coconut Genebank (ICG). * Number of countries with *in vitro* collection (this number reflects more the laboratories involved in tissue culture in coconut, where United Kingdom (Imperial College, Wye) and France (IRD/CIRAD team, Montpellier) have an important and active place).

threatened varieties, and varieties with special traits; (iii) additional diversity discovered during national explorations; and (iv) duplicates of accessions from other regions (Batugal, 1997). In addition, the ICG will undertake field evaluations and share data and germplasm with member countries using safe exchange guidelines as prescribed by FAO and IPGRI (IPGRI, 2000).

4. Conclusions

The coconut palm is a major agricultural species and is an important subsistence crop. Since the mid-20th century, a decline in productivity has occurred worldwide, despite the use of improved planting material and agronomic practices. Biotechnology and its application to coconut can create new oppor-

Table 4.1.6. State of the coconut germplasm present in the host countries of the regional coconut genebank. The state of coconut germplasm present in Vanuatu is given taking account of its interesting diversity despite the great risk of genetic erosion caused by coconut foliar disease (CFD). (Adapted from Baudouin, 1998; N'Cho *et al.*, 1998; Ramanatha Rao and Batugal, 1998.)

Ecotypes	Côte d'Ivoire Ecotype/ Accession	India Ecotype/ Accession	Indonesia Ecotype/ Accession	PNG Ecotype/ Accession	Vanuatu Ecotype/ Accession
Tall	36/20,600	68* + 34**/nc	79/4,337	17/nc	24/2,261
Semi-Tall	—	2 + 0/nc	—	—	—
Dwarf	17/4,200	16 + 12/nc	9/923	6/nc	17/1,085
Hybrids	12/nc	nc	nc	nc	nc
Indigenous		34 Tall/12 Dwarf			
Total accessions	27,962	nc	nc	nc	nc

nc, not communicated; PNG, Papua New Guinea.

*Number of ecotypes collected in different areas outside India; ** number of indigenous Indian ecotypes.

tunities in breeding, cloning, disease control and germplasm exchange/conservation. COGENT/IPGRI encourages and supports collaboration among various national coconut research groups; this is absolutely critical as there are insufficient funds to support the research needs for this crop (Hoche *et al.*, 1998b; Punchihewa, 1999; Rohde *et al.*, 1999). The development of molecular breeding tools, e.g. linkage maps and QTLs, should facilitate MAS for the recovery of hybrids with greater productivity and resistance to diseases (Cardena *et al.*, 1999). Safe exchange of germplasm can only occur if there are accurate methods for detecting and elimination of diseases.

Cryobanks for zygotic embryos are a reality (N'Nan *et al.*, 2003), and investigations based upon cryopreservation of plumules will have a great impact on storage and management of genetic resources (Hornung *et al.*, 2001; Malaurie *et al.*, 2003).

Somatic embryogenesis is promising as a means for propagating elite material and for genetic manipulation. After several decades of little success, there are now clonally propagated plants in the field (Verdeil *et al.*, 1999). The number of plantlets that have been recovered from somatic embryos remains low and

their conversion rate is unacceptable. There is a need to better understand the basic botany and biochemistry of coconut somatic and zygotic embryo development. Studies are under way that would characterize genes that are implicated in the cell cycle regulation of coconut (Sandoval, 2002; Sandoval *et al.*, 2003). Such studies together with genetic transformation (C. Oropeza, personal communication) should provide opportunities for coconut genetic engineering and improvement.

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4.2 *Elaeis guineensis* Oil Palm

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1. Introduction

1.1. Botany and history

The oil palm (*Elaeis guineensis* Jacq) genome, estimated by flow cytometric analysis (Rival *et al.*, 1997c), is 3.4×10^9 bp with a $2n = 2x = 32$ karyotype. There is a single terminal meristem, which produces an average of two leaves per month. Vertical growth rates can vary from 30 to 75 cm p.a., depending on the genotype. The root system is composed of a large number of fasciculate adventive roots. Oil palm grows preferentially in rich and temporarily damp alluvial soils (Hartley, 1988). It can reach 30 m in height, with a crown of four to five opened palmate leaves of 10–16 m in diameter, and therefore requires a large area for growth. The standard density in industrial plantations is generally 143 palms per hectare. The oil palm is a temporal dioecious species (Cruden, 1988), which displays alternate male and female flowering cycles throughout the life of the plant.

1.2. Importance

Industrial exploitation of oil palm started at the beginning of the 20th century, initially in South-east Asia, later along the Gulf of Guinea in Africa and then in tropical

America. During the latter half of the 20th century, its cultivation area extended in the Americas, mainly in the Amazon basin and along the Pacific coast of Colombia and Costa Rica. Intensive planting also occurred in South-east Asia and to a lesser extent in West Africa.

Palm oil is the second largest source of edible oil in the world after soybean (Scowcroft, 1990). It is used as a liquid cooking oil and in the manufacture of shortenings, margarines (Sudin *et al.*, 1993) and spreads (Pantzaris, 1993). Palm oil contributes approx. 20% of world oil and fat production. It is projected that the demand for oil will soon exceed supply (Oil World Annual, 2001). By the year 2020, it is expected that nearly 26% of the world's oil and fat demand will have to be met by palm oil and it will capture approx. 50% of the world's oil and fat trade (Rajanaidu and Jalani, 1995). World palm fruit production has increased tenfold since 1948: 143,389,800 t (FAOSTAT, 2004). Malaysia and Indonesia together account for > 80% of world production, with an average increase of 7% between 1995 and 2000, while production remained static in South and Central America, particularly in Colombia. The consumption of oleaginous products continues to rise due to demographic pressure, and increased domestic use of oil palm in producing countries.

1.3 Breeding and genetics

Due to the increasing demand for palm oil, the lack of suitable land and the probable increases in cultivation costs, planting material with high genetic potential is essential for the future of the industry. Planting material consists solely of *tenera* hybrids (fruits with shells of intermediate thickness), originating from crosses between *dura* (thick shell) and *pisifera* (thin shell) types, the thickness character being controlled by a monofactorial gene (Beirnaert and Vanderweyen, 1941).

1.3.1. Major breeding objectives

Breeding strategies are aimed at producing *dura* × *pisifera* hybrids with high oil productivity containing a high proportion of unsaturated fatty acids, a low growth rate and, in certain cases, resistance to diseases such as vascular wilt caused by *Fusarium oxysporum* f. sp. *elaeidis* in Africa (Hardon *et al.*, 1976). Breeding schemes now incorporate the exploitation of genetic resources able to provide resistance to diseases that are confined to specific cultivation areas, e.g. *Ganoderma* disease in South-east Asia and bud rot in South and Central America.

Oil palm breeding is based on the Sh gene, which determines three variety types (Beirnaert and Vanderweyen, 1941): *dura* (homozygous loci Sh+/Sh+) with thick-shelled fruits, *pisifera* (homozygous loci Sh−/Sh−), which is generally sterile female with shell-less fruits, and *tenera* (heterozygous loci Sh+/Sh−) with medium-thickness shells. Breeders are able to build up families of full-sib individuals that enable the evaluation of the general and specific combining abilities of their parents. Oil palm is a prime model for tropical tree crop quantitative genetics.

Since each selection cycle is approx. 10 years, genetic improvement is very slow (Jacquemard *et al.*, 1997). High heterogeneity occurs among hybrids, with some seedling trees producing 60% more oil than the average of the progeny of a given cross (Noiret, 1981). These characteristics must be considered along with the low planting density and the necessity of establishing seed orchards

for the production of commercial planting material. Oil palm improvement is labour intensive, time consuming and expensive.

1.3.2. Breeding accomplishments

Oil palm breeding is long-term (Billotte and Baudouin, 1997), and is based on reciprocal recurrent selection (RRS) (Comstock *et al.*, 1949). It is based on two groups of genotypes, and exploits their complementary yield components, i.e. group A (Deli, Angola) and group B (African populations), tested in combination with respect to each other (Meunier and Gascon, 1972). Selection is based on a relatively limited number of parents that differ in terms of origin and genetic characteristics. The genetic progress in terms of oil production is 15–18% for the second selection cycle at the Centre de Coopération Internationale en Recherche Agronomique pour le Développement – Département Cultures Pérennes (CIRAD-CP) (Cochard *et al.*, 1993) compared to the first cycle, and there should be a further 30% gain by clonal selection of the best individuals obtained from the best progenies (Baudouin and Durand-Gasselien, 1991).

2. Molecular Genetics

2.1. DNA markers

Genetic diversity in *E. guineensis* germplasm has been measured using isoenzymes (Guesquière, 1983) and restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers (Cheah *et al.*, 1991; Shah *et al.*, 1994; Jack *et al.*, 1995; Mayes *et al.*, 1996; Singh *et al.*, 1998). Both complementary DNA (cDNA) and RFLP probes have been used to characterize *Elaeis oleifera* germplasm (Barcelos *et al.*, 1999). Cheah and Wooi (1993) and Mayes *et al.* (1997) described the use of genetic markers for cultivar identification and genetic mapping, although incomplete, with 860 cM mapped and 24 linkage groups compared to 16 chromosome pairs. Singh and Cheah (1996, 2000) identified cDNA

markers of genes involved in floral development, and Rashid and Shah (1996) and Shah and Cha (2000) identified genes coding for fatty acid biosynthesis enzymes. RAPD markers for somaclonal variants (Chowdhury, 1993; Paranjothy *et al.*, 1993; Rival *et al.*, 1998) and for bud rot tolerance (Ochoa *et al.*, 1997) have been described. Pathogenic strains of *F. oxysporum* f. sp. *elaedis* have been identified with RFLP markers (Mouyna *et al.*, 1996). Purba *et al.* (2000, 2001) described the joint use of selection indices and molecular markers to optimize breeding.

2.2. Marker-assisted selection

Molecular markers are neutral with respect to the environment and can be detected at every stage of plant growth. Molecular markers indicate the presence or absence of genes and can be used to impose selection pressure on each cycle of oil palm genetic improvement to achieve greater genetic progress, e.g. molecular markers for resistance to vascular wilt and bud rot will make it possible to screen the parents and carry out marker-assisted selection (MAS) of germplasm.

Most agronomic characters are governed by several interacting genes of small effects (quantitative trait loci or QTL). Little is known about the number and effects of these genes, which leads to imprecise phenotypic selection. The MAS concept assumes a strong genetic link between the markers and loci of the genes studied, and requires prior genetic mapping of the species (Mohan *et al.*, 1997). The markers are located on the genome sufficiently densely for each locus of the genome to be strongly linked to at least one of them. Locus detection and evaluation of the effects of worthwhile genes are based on the relation between molecular polymorphism and variation in the characters studied (Charcosset, 1996).

Microsatellite markers are the basis of MAS (Fig. 4.2.1). Simple sequence repeats (SSRs) or microsatellites are tandem arrays of simple nucleotide motifs that are ubiquitous components of eucaryotic genomes (Delseny *et al.*, 1983; Tautz and Renz, 1984;

Tautz, 1989). Inherited in a Mendelian fashion (Weissenbach *et al.*, 1992; Saghai Maroof *et al.*, 1994), their hypervariable length polymorphism is revealed by the polymerase chain reaction (PCR) using flanking primers that generate co-dominant markers. According to Smith *et al.* (1997), SSR technology is reliable, reproducible, discriminating and cost effective. Development of oil palm microsatellite markers has been used for measuring genetic diversity, variety identification, pedigree analysis and genome mapping and for QTL detection for MAS (Jones, 1989; Brown, 1993; Jack and Mayes, 1993; Mayes *et al.*, 1996a,b). QTL for vegetative and yield characters and for vascular wilt tolerance based on nursery evaluations will be detected by studying the correlation between the markers and the phenotypic characters of the individuals chosen for the individual or consensus maps. The variability of QTL effects will be studied in relation to the environment, based on control progeny duplicated under different conditions. Two complementary studies have been conducted to identify AFLP markers linked to the Sh gene: (i) bulk segregant analysis (BSA) of segregant groups (Michelmore *et al.*, 1991); and (ii) genetic mapping (Billotte *et al.*, 2001). A total of 124 *Eco*RI/*Mse*I AFLP primer pairs and 88 microsatellite primer pairs have been used to analyse three *dura*, *tenera* and *pisifera* segregant groups, each consisting of DNA of eight individuals obtained by selfing a *tenera* parent LM2T. Out of a total of 9330 loci screened by the AFLP technique, an AFLP-BSA candidate marker AggCAA20 was identified and was validated by individual analyses of the DNA making up the mixes.

Billotte *et al.* (1999) described an efficient technique for building microsatellite-enriched libraries using biotin-labelled microsatellite oligoprobes and streptavidin-coated magnetic beads. This technique allowed the construction of several oil palm (GA)_n, (GT)_n or (CCG)_n enriched-libraries from total genomic as well as chloroplast DNA (cpDNA). About 200 functional SSR primer pairs have already been developed by CIRAD from microsatellite clones and have been sequenced.

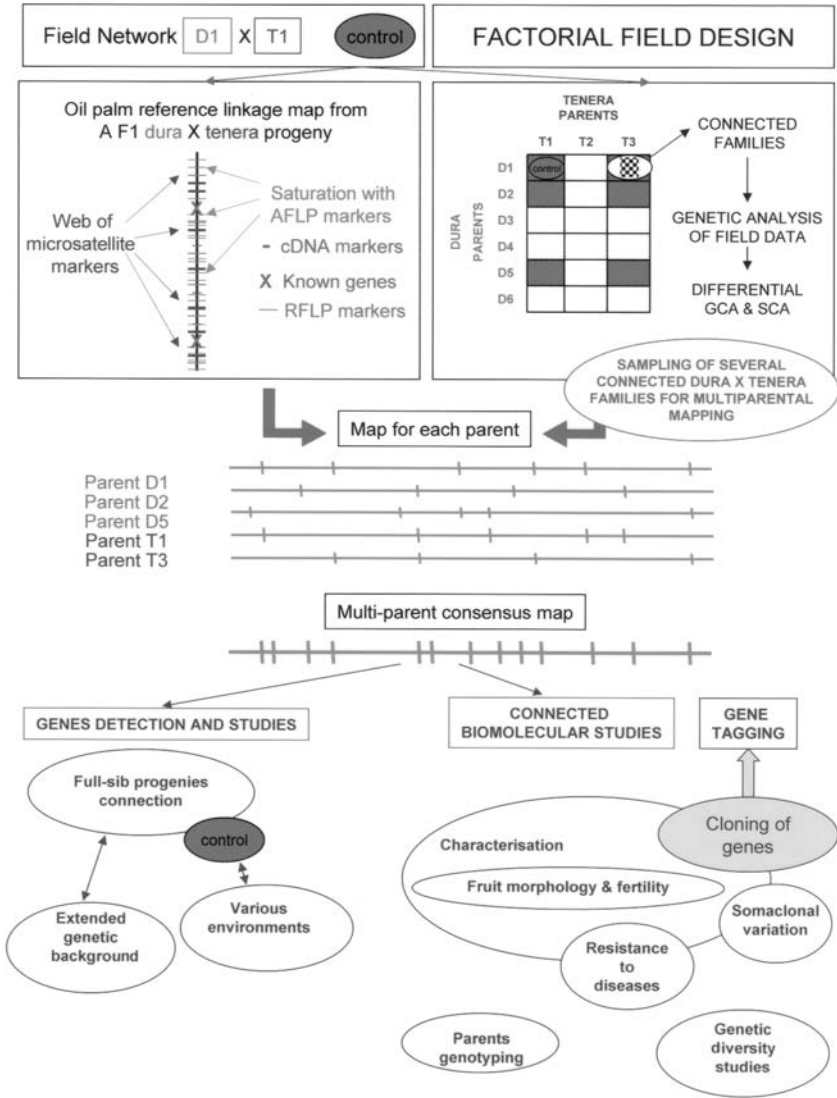


Fig. 4.2.1. Scheme of biomolecular research strategy towards marker-assisted breeding of oil palm developed at CIRAD, France.

Billotte *et al.* (2001) characterized 21 SSR loci together with primer sequences; estimates of allele size range were made, as well as expected heterozygosity in *E. guineensis* and in the closely related species *E. oleifera*, where an optimal utility of the SSR markers was observed. Multivariate data analyses indicated that SSR markers can reveal the genetic diversity within the genus *Elaeis* in accordance with known geo-

graphical origins and measured genetic relationships based on previous molecular studies (Fig. 4.2.2). High levels of allelic variability indicate that *E. guineensis* SSRs will be powerful for genetic studies of the *Elaeis* genus for variety identification and intra- or interspecific genetic mapping (Table 4.2.1). PCR amplification tests, from a subset of 16 other palm species, and allele sequence data show that *E. guineensis* SSRs

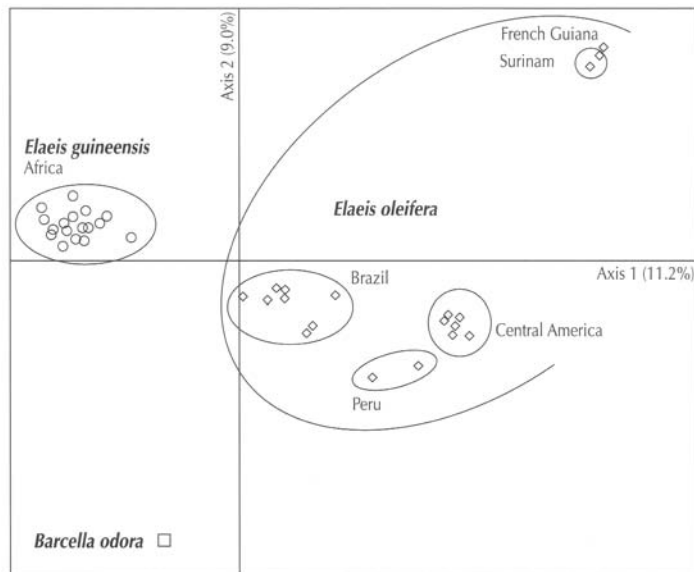


Fig. 4.2.2. Factorial analysis of correspondences performed on 20 single-locus microsatellite markers over 18 accessions of *E. guineensis* (Africa), 19 accessions of *E. oleifera* (Brazil, Central America, French Guyana, Peru and Surinam) and one accession of *Barcella odora*. Note: Axes 1 and 2 represent 20.2% of the total molecular variability.

Table 4.2.1. Type of repeats and average values of SSR allele numbers, expected heterozygosity (He) and probability of identity (PI) of 20 *E. guineensis* SSR loci in *E. guineensis* (*E.g.*) and *E. oleifera* (*E.o.*).

Sample	SSR motif	Repeats number	No. alleles <i>E.g.</i>	No. alleles <i>E.o.</i>	No. shared alleles	Total no. alleles	He			PI	
							<i>E.g.</i>	<i>E.o.</i>	<i>E.g.</i> + <i>E.o.</i>	<i>E.g.</i>	<i>E.o.</i>
9	(GA) _n	17	7.1	7.3	3.0	11.4	0.7	0.7	0.8	0.02–0.15	0.02–0.38
7	(GT) _n	10	4.3	3.9	1.6	6.6	0.4	0.4	0.6	0.02–1.00	0.09–1.00
4	(CCG) _n	6	2.7	3.5	1.7	4.5	0.4	0.6	0.6	0.11–0.40	0.12–0.23
Average	–	–	5.2	5.3	2.2	8.4	0.5	0.6	0.7	0.3	0.2
Polymorphic markers							80%	95%	100%		

are putative markers for all palm taxa. In addition, phenetic information based on SSR flanking region sequences makes *E. guineensis* SSR markers a potentially useful molecular resource for study of palm taxa phylogeny.

Field trials consisting of several populations to be used for MAS are to be established at different locations, consisting of 5000 genotypes and involving at least 20 parents (full-sib families). The objectives are to test the accuracy of molecular markers on multi-parent and connected full-sib families from extended genetic backgrounds and are

dedicated to a wider identification of intra- and inter-population QTL diversities. Results will be integrated into breeding programmes in the form of an applied multi-character marker-assisted breeding strategy for worthwhile genes. Various parents will be used, including individual palms suspected or known to be resistant to diseases caused by *Ganoderma* and *F. oxysporum*. Field trials will test interspecific back-crosses of the first or second generation for MAS and introgression of important *E. oleifera* characters into oil palm: low height, high oil quality and resistance to *Ganoderma*, *Fusarium* wilt and

bud rot. Molecular marker data will be associated with selected phenotypic characters to define and apply MAS.

Molecular markers will be used to select *dura*, *tenera* or *pisifera* individuals at the nursery stage. In the same way, it will be possible to detect and plant *pisifera* individuals in male parent plots for *tenera* seed production. Using gene markers, it should be possible to evaluate the genetic value of *pisifera* individuals, which has been impossible in the field due to their female sterility. Effective management of the genetic variability of *E. guineensis* will be possible, with field testing of *tenera* × *tenera* progenies, of which only *tenera* individuals will be retained. The most worthwhile parents will be genotyped and survey populations will be integrated into the breeding programme.

Biotechnology can be fully integrated into a coordinated programme aimed at selecting, multiplying and disseminating genetically improved material to the industry. Studies in the medium term will address the following priorities: (i) the molecular dissection of the chromosomal region surrounding the major gene *Sh*, coding for the existence or lack of a shell, in order to clone and label the related genes for fruit morphology and fertility, i.e. degree of shell lignification, kernel volume in *dura* and *tenera* varieties, female sterility of *pisifera* genotypes; (ii) characterization, cloning and tagging of vascular wilt tolerance genes; (iii) molecular studies of genetic diversity of oil palm, which will be based partly on using QTL markers and will determine the potential for applying natural diversity, particularly to predict heterosis; and (iv) search for tolerance of bud rot by genetic mapping of tolerance genes in *E. oleifera* (Le Guen *et al.*, 1991) for introgression into *E. guineensis* by MAS.

2.3. Genetic mapping

In addition to providing further knowledge of the genome, reference maps will enable markers and parents to be selected. A control *dura* Deli × *tenera* La Mé F₁ progeny has been chosen (cross DA10D × LM2T). The

homologous and heterologous markers mapped will primarily be co-dominant, locus-specific and easily transferable from one population to another (microsatellites, genomic RFLP, cDNA markers and expressed sequence tag (EST) markers). Highly polymorphic microsatellite markers will make up the web of the reference genetic map; they are effective in genetic diversity studies, notably of oil palm (Shah and Nyuk, 1996), and suitable for genome mapping (Akagi *et al.*, 1996). These markers will be topped up with RFLP and cDNA 'anchor points'. AFLP markers will be used to saturate the reference linkage map.

Muranti (1996, 1997) showed that a multi-parent consensus map, established with several parents and full-sib families linked within a diallel or factorial design should be effective in the search for QTL suitable for use in MAS. In addition to ensuring more accurate detection compared to a single two-parent map, it also enables an evaluation of the effects of QTL depending on their type (additive, dominance) and of different genetic bases. Such a multi-parent map is being established by CIRAD and PT SOCFINDO (Indonesia), using microsatellite and cDNA markers on an existing factorial genetic design already observed for most vegetative and production characters. The map population consists of about 600 palms and several *dura* × *tenera* full-sib families, including the reference map population, obtained from different La Mé, Deli and Yangambi parents. All individual maps of the system will be connected between themselves by common parents and by co-segregating microsatellite or cDNA/EST markers.

An F₁-type progeny (*dura* × *tenera*) of 90 individuals obtained from a DA115D × LM2T cross was chosen for genetic mapping and the *Sh* gene in the LM2T parent. The LM2T genetic map, constructed with MAPMAKER at log of odds (LOD) score = 5.0 and *r* = 0.3, distributed a set of 149 AFLP or microsatellite markers in 15 linkage groups, three pairs and six unlinked markers (Fig. 4.2.3). The number of linkage groups, close to the number *n* = 16 pairs of chromosomes of the plant, and a total map

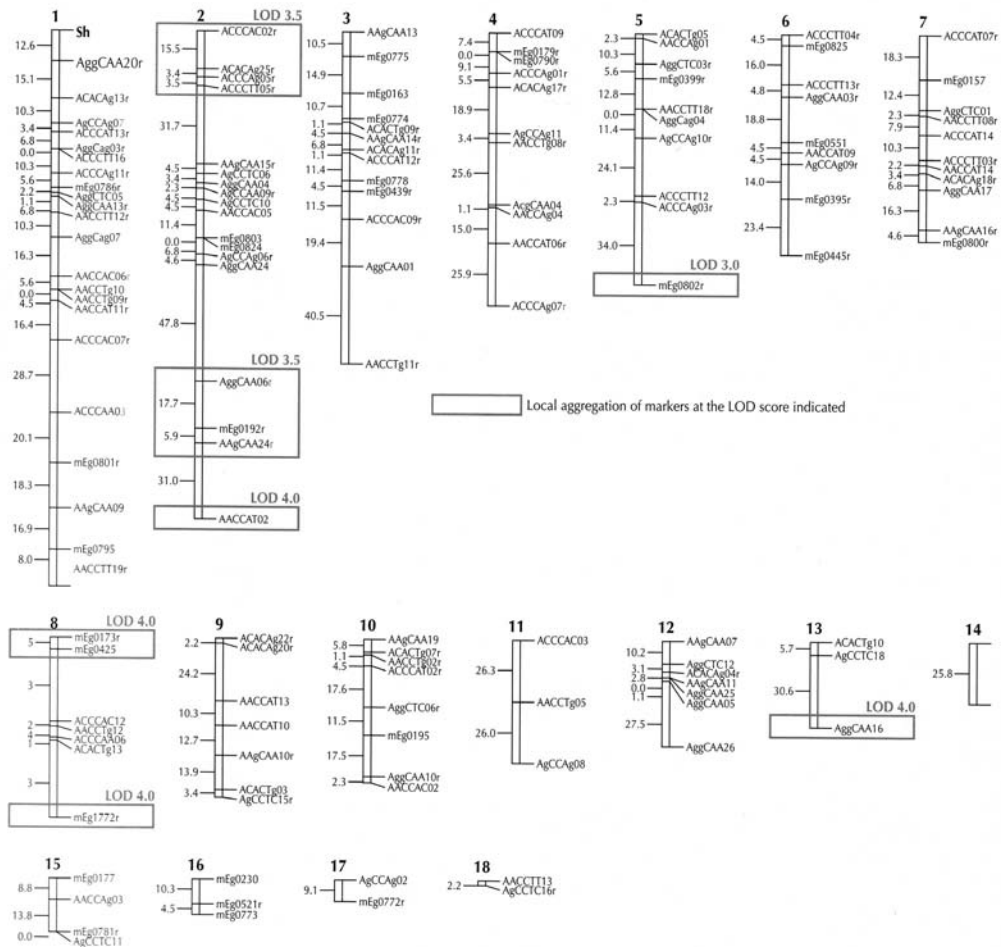


Fig. 4.2.3. AFLP and microsatellite oil palm linkage map of the *tenera* LM2T parent, built with the MAPMAKER 3.0 software (LOD score = 5.0; $r = 0.3$). Note: the *Sh* gene and its AFLP-BSA marker are mapped on the linkage group number 1.

size of 1355 cM indicate relatively good coverage of the genome. The AFLP-BSA marker, which had revealed a co-dominance reading of the *Sh*⁺ gene in the *dura* and *tenera* phenotypes, was likewise mapped in LM2T. The gene *Sh* and its AFLP-BSA marker were mapped on to the longest linkage group of the map (219.5 cM) at 7.2 cM or 12.6 cM according to JOIN-MAP or MAPMAKER, respectively. The study showed the pertinence of BSA analysis in the search for molecular markers of

the *Sh* gene, and the complementarity of the genetic mapping approach. The AFLP-BSA marker and the genetic map of LM2T are an important step towards MAS of the *Sh* gene, and of other genes of agronomic interest.

Rance *et al.* (2001) have recently developed an oil palm RFLP marker map which has enabled the initiation of marker-based QTL mapping studies. The QTL mapping analysis was carried out by interval mapping and single-marker analysis.

2.4. Functional genomics

The differential display reverse transcription PCR (ddRT-PCR) technique (Liang and Pardee, 1992) has been used to identify putative early markers for clonal uniformity by comparing mRNA abundance between two or more RNA samples. The isolation, validation and characterization of expression markers of the *mantled* abnormality involves a number of stages. The *mantled* abnormality has been studied using cultures derived from: (i) a normally flowering seed-derived palm; (ii) an abnormal ramet palm previously cloned from the latter; and (iii) a normal ramet palm previously cloned from the original seed-derived palm. Gene expression has been measured at three different *in vitro* stages, i.e. embryogenic culture, polyembryonal mass (PEM) maintenance phase and leafy shoot stages, with emphasis on the embryogenic culture and leafy shoot stages.

Differential display provides candidate markers, which must be validated and characterized using a range of different molecular techniques. Data obtained from the leafy shoot stage are presented in Table 4.2.2. Putative expression markers of the *mantled* abnormality representing different *in vitro* stages (embryogenic culture, somatic embryos, leafy shoots and greenhouse-harvested leaves) have been characterized. One of these genes, called *EGAD1*, codes for a protein belonging to the family of the plant defensins (Tregear *et al.*, 2001). An over-accumulation of the transcripts of the *EGAD1* gene was observed in variant *in vitro* cultures at the callus stage, in contrast to normal cultures. The *EGAD1* marker has been jointly patented by CIRAD and the Malaysian Palm Oil Board (MPOB). A collection of systematically sequenced EST cDNA clones

is currently being established in order to study the function of the apical meristem. A collection of genes expressed in the oil palm shoot meristem within specific tissues, at certain developmental stages or in response to environmental conditions, will be identified. An extensive catalogue of oil palm genes will be screened either on the basis of their sequence affinities (similarity to known genes of interest) or by using high-throughput macro- or microarray screening to examine their expression patterns.

2.5. Gene cloning

2.5.1. Desaturases

Plants have two main types of desaturases, a soluble plastid Δ^9 stearoyl-acyl carrier protein (ACP) desaturase and membrane-bound desaturases which are involved in C12 and C15 unsaturation. Full-length clones of Δ^9 stearoyl-ACP desaturase have been isolated from a 15-week mesocarp cDNA library (Siti Nor Akmar *et al.*, 1997, 1999). At least four members of the Δ^9 stearoyl-ACP desaturase gene family are present in the oil palm. The coding sequences of the genes were identical; however, all four genes had 3' untranslated sequences that were different. Northern analysis using specific probes that were constructed based on the 3' untranslated sequence showed that two clones, designated pOP-SN16 and pOP-SN20, were highly expressed in mesocarp, kernel and leaf at different stages of development. These genes probably have housekeeping functions, i.e. membrane lipid synthesis. Expression of two other clones, designated pOP-SN18 and pOP-SN19, were specifically correlated with oil synthesis in mesocarp (Siti Nor Akmar, 1999).

Table 4.2.2. Summary of differential display marker data for leafy shoot material.

No. of potential markers identified by differential display	46
No. of individual cloned cDNA fragments obtained	58
No. of cDNAs producing differential signals in first stage of Northern retesting	13
No. of cDNAs producing stronger Northern signal in normal tissue	6
No. of cDNAs producing stronger Northern signal in abnormal tissue	7
No. of cDNAs producing consistent signals for several genotypes (confirmed to date)	3

E. oleifera mesocarp contains more unsaturated fatty acids than *E. guineensis*. A stearyl-ACP desaturase cDNA clone of *E. oleifera* was isolated using the stearyl-ACP desaturase gene from *E. guineensis* as a probe (Siti Nor Akmar, 1999). The nucleotide sequences of the *E. guineensis* and *E. oleifera* cDNA clones are nearly homologous (> 99%) and well conserved during the evolution of these species (Siti Nor Akmar *et al.*, 2001). Two isoforms of the $\Delta 9$ stearyl-ACP desaturase gene have been recently isolated (Shah *et al.*, 2000).

2.5.2. Acyl carrier proteins

Active oil synthesis in mesocarp begins approx. 15 weeks after anthesis (WAA) and terminates at approx. 21 WAA (Oo *et al.*, 1985). Certain enzymes or proteins involved in fatty acid biosynthesis are most active at the period of oil synthesis while the regulatory proteins, which are involved in switching on or increasing the level of gene expression, may be present at the start of or just prior to the period of active oil synthesis. ACP is temporally specific for oil synthesis. Besides acting as a temporally specific marker, the ACP transit peptide could possibly be used for targeting cytoplasmic enzymes into chloroplasts or plastids.

Efforts have been directed towards isolation of the oil palm ACP cDNA clones prior to isolation of genomic clones for identification of the gene promoter sequences. Using a partial rice ACP gene (C1555) as a probe, several partial clones and one full-length clone of ACP have been isolated by screening a 15-week mesocarp cDNA library by plaque hybridization (Rasid *et al.*, 1999). All clones were sequenced, showing that there were at least two members of the ACP gene family. A partial clone designated pACP1 has a 3' untranslated non-coding region which is different from that of the full-length clone (pACP3) and the other partial clones. The coding region of pACP3 is identical to those of the partial clones except for pACP1, which showed only 93% homology with pACP3. The pACP3 sequence showed approx. 70% homology with barley and wheat ACP sequences. The full-length clone was 701 bp;

the open reading frame comprised 144 amino acid residues, of which 61 constitute the transit peptide and 83 make up the mature protein. A full-length ACP cDNA clone from *E. oleifera* has also been isolated and analysed (Rasid *et al.*, 2001).

2.5.3. Acyl-ACP thioesterase

Two sequence-specific primers were designated ACO1 and ACO2 based on a 130-base pair thioesterase sequence from the oil palm mesocarp. Using PCR, a 750 bp fragment from a 15-week mesocarp cDNA library and an 800 bp fragment from a 17-week mesocarp cDNA library were consistently obtained when using ACO1 and T7 primers (which would give the 3' end sequence). When using ACO2 and T3 primers (which would give the 5' end sequence), a 700 bp fragment from a 15-week cDNA library and a 350 bp fragment from a 17-week cDNA library were obtained. ACO2 and T3 indicated that there were two clones in the library that extended as far as the ACO1/2 region, and no clones were present with inserts > 1500 bp. The 750 bp and 800 bp fragments were used to probe a 15-week mesocarp cDNA library. Three plaques hybridized to both 3' and 5' probes. These cDNA clones were purified and transformed into *Escherichia coli* DH5 α . Digestion analysis showed inserts of approximately 1450 bp, derived from a single cDNA clone. Sequence analysis showed the cDNA (designated pHA-3) to be incomplete, extending from the coding region to the poly(A) tail, with the 5' end of the insert located within the transit peptide sequence. The 3' region is 360 bp. This sequence, corresponding to the mature protein, is 66% identical to *Cuphea hookeriana* Ch FatB1 and 35% identical to *Garcinia mangostana* Garm FatA1, indicating that this clone belongs to the FatB (16:0) (Kinney, 1998) type of acyl-ACP thioesterase. A full-length *FatB* thioesterase gene has been isolated and is over-expressed in *E. coli*. The cloned enzyme can hydrolyse medium-chain and long-chain acyl-coenzyme As (CoAs). Four full length clones of thioesterase have been isolated (Murase *et al.*, 2000).

2.5.4. KAS II

A nearly full-length KAS II clone has been isolated from oil palm mesocarp (Umi Salamah, personal communication in Siti Nor Akmar *et al.*, 2001). The information on KAS II in the database is very limited; however, the full-length KAS II gene has been isolated (Murase *et al.*, 2000).

2.5.5. Oleoyl-CoA desaturase

Biosynthesis of oleic acid is restricted to the plastid. Desaturation of oleic acid requires CoA as a cofactor and takes place in the cytosol. This reaction is catalysed by the action of oleoyl-CoA desaturase. The higher correlation between KAS II and linoleic acid (C18:2) compared to oleic acid (C18:1) suggests that increasing the activity of KAS II may result in spillover of the increased oleic acid to linoleic acid. Genetic manipulation for high oleic acid may therefore require the silencing of this gene. Efforts are in progress to isolate this gene from the oil palm (Siti Nor Akmar *et al.*, 2001), and a cDNA clone for oleoyl-CoA desaturase has been isolated (Murase *et al.*, 2000).

2.5.6. Isolation of tissue-specific promoters

In order to genetically engineer the oil palm to produce high oleic acid, the target genes should be expressed in the mesocarp. Various approaches are being used to isolate cDNAs from the mesocarp, including differential screening (Siti Nor Akmar *et al.*, 1995), subtractive hybridization (Siti Nor Akmar *et al.*, 1996), differential display (Nurniwalis and Siti Nor Akmar, 2000) and DNA microarray (Siti Nor Akmar *et al.*, 2001). A cDNA library of oil palm mesocarp has been constructed, using size-fractionated mRNA from fruit mesocarp 15 WAA (Siti Nor Akmar *et al.*, 1995). Approx. 200,000 cDNA clones have been obtained and 60% of them are recombinant, based on β -galactosidase assay. Insert sizes of 0.4–2.5 kbp have been obtained. Mesocarp-specific cDNA clones have been screened using subtracted cDNA probes, which were prepared by hybridizing labelled first strand 15-week mesocarp cDNA to 5-week mesocarp mRNA. The probes have been used to obtain highly abundant and development-specific cDNA

clones. Eight putatively development-specific clones have been identified (Siti Nor Akmar *et al.*, 1996). A mesocarp-specific gene has been isolated and the gene is highly abundant in mesocarp tissue with no expression in other tissues (Siti Nor Akmar, 1999). The Universal Genome Walker Kit was used to isolate the promoter for the above gene. The promoter was attached to a green fluorescence protein (GFP) gene and showed expression only in the mesocarp (Zubaidah and Siti Nor Akmar, 2000; Siti Nor Akmar *et al.*, 2001). A mesocarp and species-specific cDNA clone that codes sesquiterpene synthase has also been isolated (Shah and Cha, 2000). Isolation of a mesocarp-specific promoter has also been carried out (Shah and Cha, personal communication).

2.5.7. Kernel-specific promoter

A cDNA clone coding for the glutelin gene has been isolated through sequencing random cDNAs from cDNA libraries and transcript profiling. The full-length cDNA sequence of this gene of 1.6 kb was obtained by 5'-RACE using total RNA from the kernel. The coding sequence shares 94% homology with one of the two glutelin gene sequences (accession nos. AF193433 and AF261691) (Cha and Shah, 2001). Southern analysis has revealed that there are several copies of the glutelin gene in the oil palm genome (Siti Nor Akmar and Cheah, 2000). Northern analysis indicates that it is highly expressed in the kernel and endosperm with no expression in the embryo. Since the endosperm is the site of oil synthesis, the promoter of this gene could control expression of introduced genes for modifying kernel oil composition (Siti Nor Akmar *et al.*, 2000). The genomic clones have been isolated using the genome walking approach, which also has been used to clone the promoter sequence. A 2.0 kb PCR product has been amplified and analysed (Siti Nor Akmar *et al.*, 2001). Three isoforms of glutelin gene have been isolated (Cha and Shah, 2001).

2.5.8. Leaf-specific cDNA

A few putative leaf-specific cDNAs have been identified by differential display and differen-

tial screening of a leaf cDNA library (Chan *et al.*, 2000). A homologous cDNA fragment has been amplified by RT-PCR. Its leaf-specific expression has been confirmed by Northern blot analysis (Chan, personal communication, in Siti Nor Akmar *et al.*, 2001).

3. Somatic Cell Genetics

3.1. Regeneration

Oil palm cannot be vegetatively propagated by conventional means, despite attempts to reverse floral bud differentiation and manipulation of vivipary (Chevalier, 1910; Henry, 1948; Davis, 1980). The *in vitro* culture of apices was attempted with young palms (Staritsky, 1970), but the results were unsatisfactory. Excision of the apex results in death of the mother tree. Constraints of classical oil palm breeding have stimulated development of a fairly reliable *in vitro* regeneration protocol which would allow: (i) exploitation of the variability among *tenera* hybrids by cloning elite individuals (Noiret, 1981); (ii) increased production of high-quality seeds by cloning the best male parents (*pisifera*), since pollen production can be a limiting factor (Hartley, 1988; Krikorian, 1989); (iii) exploitation of *E. guineensis* × *E. oleifera* interspecific hybrids, a limited number of which are fertile, but which can show tolerance of pests and diseases (Meunier, 1975); (iv) production of biclonal seeds from somatic embryogenesis-derived parents (Hartley, 1988); and (v) regeneration of genetically engineered material bearing useful agronomic traits (Rival, 2000).

3.1.1. Somatic embryogenesis

Somatic embryogenesis has been developed for micropropagating elite oil palm selections and for genetic manipulation using somatic cell genetic approaches. Studies were initiated by Unilever Plantations (UK), Harrison and Crossfields Plantations group (Malaysia) (Corley *et al.*, 1977; Smith and Jones, 1970) and IRHO/ORSTOM in France and Côte

d'Ivoire (Rabéchault *et al.*, 1970; Noiret, 1981), and were intended to complement in-house breeding strategies for multiplying elite germplasm for commercial use. Due to the important commercial applications of the results, only limited technical information was published (Krikorian, 1989; Blake, 1990). Later, in Malaysia, several teams developed large-scale research programmes, notably the Palm Oil Research Institute of Malaysia (PORIM), now MPOB (Paranjothy and Othman, 1982). Wooi (1990) estimated that there have been as many as ten commercial laboratories for micropropagating oil palm. Many elite selections have been regenerated, including *E. guineensis* × *E. oleifera* interspecific hybrids (Duval *et al.*, 1997).

Rival (2000) recently reviewed regeneration protocols for oil palm of different genetic origins. The frequency at which proliferating embryogenic cultures, i.e. PEMs, are obtained, enabling mass production, is c. 40%, and is currently the major stumbling block for large-scale ramet production for many selected ortets. The regeneration of oil palm by somatic embryogenesis comprises four steps: induction, embryogenesis, shoot development and rooting. *In vitro* culture of this plant is characterized by extended delays of up to 2 years between sampling of the primary explants and acclimatization of the regenerated plantlets, low efficiency of some steps of the process and production of polyphenols, whose influence on regeneration is poorly understood.

Induction. With adult palms, induction of cultures involves immature leaves (Pannetier *et al.*, 1981), roots and possibly young inflorescences (Wooi, 1990). Despite the limited information available, it is recognized that induction occurs in the presence of auxins on full- or half-strength Murashige and Skoog (1962)-derived basal medium (MS) (see Wooi, 1990) and is supplemented with vitamins, 0.5 to 1 mg/l casein hydrolysate and 20 to 30 g/l sucrose or glucose. The pH ranges between 4.5 and 5.7 but seems to have little influence on the success (Krikorian, 1990). Various auxins are employed: naphthaleneacetic acid (NAA),

2,4-dichlorophenoxy-acetic acid (2,4-D) and, less frequently, 2,4,5-trichlorophenoxy-acetic acid (2,4,5-T) or 2,4,5-trichlorophenoxy-propionic acid (2,4,5-TP) at 10^{-6} and 10^{-4} M. The highest auxin concentrations are always used in the presence of activated charcoal, which adsorbs up to 99% of the growth regulators present in the medium. Cytokinins, including kinetin, benzyladenine (BA) and 2-isopentenyladenine (2iP), are sometimes added at very low concentrations. The auxin/cytokinin balance is very high (c. 20) (Duval *et al.*, 1995b) and may not be essential. Activated charcoal limits the diffusion of phenolic compounds and the darkening of both media and explants during induction, although browning is not a negative factor with oil palm (Wooi, 1990). The cultures are generally maintained in darkness at 25–30°C and the time necessary for the appearance of cultures is 8 and 16 weeks, depending on the type of explants and the genetic origin of the material.

Maintenance. Somatic embryogenesis is always indirect, with an intermediary callus phase. The calluses are transferred to a medium for embryogenesis, generally under light conditions ($45 \mu\text{mol}/\text{m}^2/\text{s}$). Auxin concentration is generally lower than that used during induction: 2.5×10^{-6} and 2×10^{-4} M of either 2,4-D or NAA. Cytokinins (BA, kinetin, 2iP) can be added to the medium at low concentrations (10^{-7} to 0.5×10^{-5} M) (see Wooi, 1990). Somatic embryos appear with almost all clones, but generally at low frequency. Experiments at the La Mé Research Station (Côte d'Ivoire) showed that, after 50 months in culture, 95% of the cultures have produced at least one embryo and 50% of them are PEM cultures, which can be used for mass production of plantlets.

The establishment of embryogenic suspensions has been reported (de Touchet *et al.*, 1991; Teixeira *et al.*, 1995) for mass production of somatic embryos (Duval *et al.*, 1995a,b; Aberlenc-Bertossi *et al.*, 2000, 2001 see also Bajaj, 1991). Embryogenic suspensions are established from friable, embryogenic cultures, which are isolated from nodular compact calluses (Duval *et al.*, 1995a). The basal medium consists of MS

macroelements, Nitsch's microelements (1969), Morel and Wetmore's vitamins (1951) and 100 mg/l sodium ascorbate. Suspensions are transferred monthly on basal medium supplemented with 20 g/l glucose, 30 mg/l adenine sulphate, $4.44 \mu\text{M}$ BA, $450 \mu\text{M}$ 2,4-D and 1 g/l activated charcoal.

Suspensions are prepared by inoculating embryogenic cultures in liquid medium, and by selection of meristematic nodules that proliferate in the presence of 2,4-D. Between 10 and 12 mg/l of PEMs are inoculated into medium containing $452.5 \mu\text{M}$ 2,4-D and 2 mg/l activated charcoal. Histological studies indicate that the proliferating PEMs are very homogeneous. To date, embryogenic suspensions have been successfully isolated for > 50 different clonal lines. The optimum concentration is c. 10^5 PEMs per litre with a multiplication factor of $4 \times$ per month. Sondahl (1991) reported the successful growth of suspension cultures in bioreactors. Implementation of production strategies based on the large-scale use of embryogenic suspensions has been severely hampered by the critical problem of somaclonal variation. Subculture to auxin-free medium is essential to stop proliferation of PEMs and to initiate somatic embryo development.

Development and maturation. Embryo development is achieved following the transfer of cell clusters on to a plant growth regulator-free liquid medium containing the basal medium supplemented with 30 g/l sucrose and 0.5 g/l casein hydrolysate. The development of somatic embryos occurs after sieving (mesh size = 1 mm) and plating on to a semi-solid medium of the same composition. The smallest PEM fraction which is capable of developing into individual plantlets is transferred into growth regulator-free liquid medium. Embryo maturation is achieved by spreading the small PEM fraction on to semi-solid medium, at a rate of 0.05 ml packed cell volume (PCV) per Petri dish, resulting in differentiation of c. 300 somatic embryos per dish. Cultures are grown under 12 h light at $45 \mu\text{mol}/\text{m}^2/\text{s}$ at 27°C. PEMs differentiate as proembryos, i.e. globular embryos with a protoderm, after

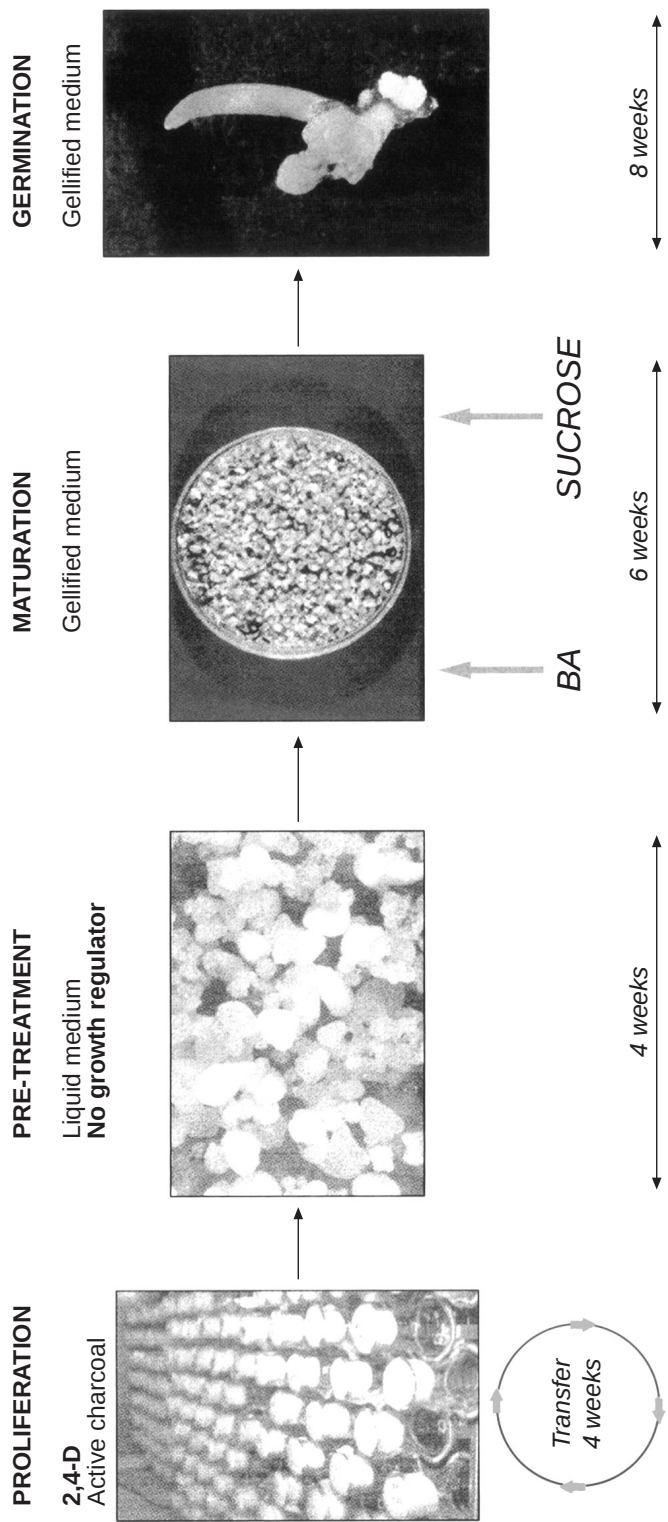


Fig. 4.2.4. Oil palm micropropagation through embryogenic suspensions (CIRAD/IRD protocol).

4 weeks. The differentiation of both shoot and root meristems occurs after 6 weeks, although only root development is commonly observed. The addition of 5 μ M BA to maturation medium can result in significantly greater shoot development, with a concomitant halt in root development. The production scheme is indicated in Fig. 4.2.4.

Somatic embryos produced from embryogenic suspensions do not develop normally and lack a quiescent phase (Aberlenc-Bertossi *et al.*, 1999). Using zygotic embryo development as a reference, physiological and biochemical characteristics of somatic embryo maturation have been studied. Changes in several key molecules involved in acquisition of desiccation tolerance, i.e. oligosaccharides and abscisic acid (ABA), and in vigour of regenerated plantlets (such as storage proteins) have been investigated. As a result, culture conditions have been modified to enhance somatic embryo maturation.

Zygotic embryos of oil palm are desiccation-tolerant and seeds can be stored for 2–3 years. Dry matter, water content, sugar and ABA contents have been investigated during zygotic embryo development. Embryo dry weight (DW) increases between 3 and 4 months post anthesis (mpa) and is then stable until shedding (c. 6 mpa). Embryos undergo partial desiccation, but water content is high at maturity (c. 1.5 g H₂O/g DW). Desiccation tolerance is significantly correlated with embryo DW and water content and tolerance is acquired at approx. 3.5 mpa. The acquisition of desiccation tolerance between the 3rd and the 4th month is associated with sugars (fructose, glucose, raffinose, stachyose and sucrose) and ABA biosynthesis (Aberlenc-Bertossi *et al.*, 1995, 2001). Sucrose stimulates dehydration of somatic embryos, increases DW and enhances somatic embryo desiccation tolerance. Exogenous ABA has no effect on somatic embryo DW and water content but lowers monosaccharide content (Aberlenc-Bertossi *et al.*, 2001). Sucrose content increases and raffinose can be detected after extended culture on ABA-containing medium; somatic embryo desiccation tolerance is enhanced and shoot emission is inhibited.

Storage proteins that accumulate during oil palm embryo development have been characterized (Morcillo *et al.*, 1997). Only water- and low salt-soluble proteins, with respective sedimentation coefficients of 2S and 7S, were detected in mature embryos. The various protein classes identified were characterized by electrophoresis and amino acid composition analysis. The 2S proteins comprise polypeptides of 22 kDa and 19 kDa, which are acidic (pI < 6) and basic (pI > 9), respectively. The 7S proteins predominate and are heterogeneous oligomers (M_r of 156 and 201), comprising a polypeptide triplet of M_r between 45 and 65 with no disulphide bonds. Their amino acid composition is broadly similar to that of the 7S proteins of other monocotyledonous embryos, but differs from that of the legume 7S vicilins. Histological examinations and electrophoresis showed that the 2S and 7S proteins appear at the 3rd month after fertilization, no qualitative changes being detected up to the 6th month of embryo development.

The accumulation of 7S globulins was studied in zygotic and somatic embryos (Morcillo *et al.*, 1998). Antibodies raised against these proteins were used for their detection by Western blotting and quantification by enzyme-linked immunosorbent assay (ELISA). In zygotic embryos, the 7S globulins were found to accumulate mainly between the 3rd and the 4th months after anthesis, corresponding to the end of the embryo growth. They constitute 10% DW and 50% of soluble protein content. The amounts of soluble protein and 7S globulins in somatic embryos were found to increase rapidly during the early stages of development, but were almost 80 times lower than in zygotic embryos. In somatic embryos, 7S globulins represented 0.3% DW and 4% of soluble proteins. After 22 days of development, protein content declined slowly. The *in vitro* production of 7S globulins (and more generally salt-soluble proteins) was improved by the addition to the culture medium of glutamine, arginine, sucrose and ABA, the effects of these components being additive (Morcillo *et al.*, 1999).

Morcillo *et al.* (2001) have attempted the characterization and expression analysis of the oil palm *GLO7A* gene encoding a 7S globulin protein. To investigate the regulation of 7S globulin gene expression in both zygotic and somatic embryos of oil palm, we isolated a cDNA clone, *GLO7A*, for use as a probe in Northern hybridization studies. The nucleotide sequence of *GLO7A* cDNA reveals that it encodes a polypeptide of 572 amino acids (66 kDa) sharing significant sequence similarities with various vicilin-like proteins of both dicotyledonous and monocotyledonous plants. Northern hybridization analysis shows that 7S globulin mRNA accumulation in zygotic embryos is temporally regulated with a profile essentially the same as that observed at the protein level. In somatic embryos, 7S globulin proteins were found to occur in amounts approximately 80 times lower than those in zygotic embryos. This lack of 7S globulin protein accumulation in somatic embryos is mirrored by a low accumulation of the *GLO7A* mRNA. The *in vitro* production of 7S globulins (and more generally salt-soluble proteins) is improved by the addition to the culture medium of arginine, sucrose and ABA, the effects of these three components being additive. We also performed parallel studies on mRNA and protein abundance. Our studies of transcript accumulation suggest that ABA and sucrose act directly on mRNA synthesis or stability; however, it appears that there are also translational or post-translational regulatory factors which limit protein accumulation in somatic embryos. To assess whether *GLO7A* gene expression might be modulated by *cis*-acting promoter elements related to those found in other plants, the *GLO7A* gene promoter was cloned and sequenced. Two motifs resembling ABREs and one motif resembling a seed-specific promoter element were identified within the 5' flanking sequence.

These results should enable us to define optimum *in vitro* culture conditions for improved tolerance of desiccation and accumulation of storage proteins. Further investigations will be undertaken to improve maturation protocols. Strategies for plant delivery based on the 'artificial seeds' concept will ultimately be developed.

Plant recovery. Rooting of shoots varies considerably, and no rooting occurs in the absence of exogenous auxins. Rival *et al.* (1997) evaluated gaiacol-peroxidase activity as a marker of *in vitro* rooting. Peroxidase activity peaked between 10 and 14 days under optimum conditions (see review by Gaspar *et al.*, 1992), and shifted in time when rooting was low. Peroxidase activity revealed a significantly greater heterogeneity in batches with a low rooting success rate (> 50%). A single rooting step is used, with an auxin treatment applied over a much longer time (8 weeks) with 2.7–5.4 μM NAA.

Acclimatization losses severely impact production costs (Rival *et al.*, 1994, 1996, 1997b, 1998a), because losses occur at the ultimate stage of the process. Photochemical activity, CO_2 exchange and carboxylase enzymatic activities were studied during PEM proliferation, somatic embryo maturation, shoot development (first and second caulogenesis cycles) and rooting (Rival *et al.*, 1997d). *In vivo* chlorophyll fluorescence measurements indicated that the maximum photochemical activity of photosystem II (PSII) was very low in the maintenance phase and strongly increased in later development stages, reaching an activity very close to that in acclimatized plants. The quantum yield of photosynthetic electron transport was similar except that a marked depression of electron transport activity was observed in the rooted plantlets. CO_2 exchange measurements showed that absolute levels of *in vitro* photosynthesis were low.

Photosynthetic activity was also investigated by focusing on the activities of two of the primary enzymes of CO_2 fixation, phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) and ribulose 1,5-bisphosphate carboxylase (RubisCO; EC 4.1.1.39), throughout somatic embryo development. The PEPC:RubisCO ratio progressively decreased, due to a substantial depletion of PEPC activity. Specific RubisCO activity was altered, except for a transient increase during the first caulogenesis stage on semi-solid medium. The relative amount of RubisCO activity increased during somatic embryo development (from 3.2% in proliferating PEMs to 38.8% in second-cycle shoots), and then decreased during rooting (26.4%).

Growth parameters (total fresh weight (FW), relative foliar FW and number of expanded leaves) and biochemical characteristics (total soluble protein and chlorophyll content, specific PEPC, RubisCO activities and relative RubisCO content) were studied (Rival *et al.*, 1998a). The PEPC/RubisCO ratio of *in vitro* plants was unaffected during transplantation to the greenhouse and remained at the same level (c. 0.05) as in *in vitro* leaves. At 60 days after *ex vitro* transplantation, chlorophyll and soluble protein contents and PEPC/RubisCO ratio were similar in seedlings and *in vitro*-propagated plants, but growth characteristics were markedly different. Relative RubisCO amounts were comparable (c. 230 mg/g protein) in leaves from *in vitro*-grown and acclimatized *in vitro* plants and were lower than in greenhouse-grown plants (350 mg/g protein) (Rival *et al.*, 1996).

Carboxylase activities, photochemical activity and CO₂ exchange indicate that photosynthetic activity could be measured as early as the first caulogenesis step, showing a noticeable increase during the second stage and less photosynthetic activity during root growth. *In vitro*-grown leaves of oil palm contribute to autotrophy and therefore are important in acclimatization (Grout, 1988). Acclimatization losses are probably due to poor rooting and/or to a poor management of the *in vitro* environment.

3.2. Genetic manipulation

3.2.1. Somaclonal variation

Approximately 5% of somatic embryo-derived oil palms are abnormal with respect to floral development, involving an apparent feminization of male parts in flowers of both sexes, i.e. the *mantled* phenotype (Corley *et al.*, 1986; Rival, 2000). This may result in partial or complete flower sterility, thus directly affecting oil production. Several potential biochemical markers of the *mantled* abnormality in oil palm have been investigated, including polypeptide patterns (Marmey *et al.*, 1991) and endogenous cytokinins (Maldiney *et al.*, 1986; Besse *et al.*, 1992); however, the usefulness of such markers on a large scale is questionable, due to lack of reproducibility and high cost.

The *mantled* abnormality is epigenetic. Reversion to a normal floral phenotype can occur in the field over time, leading to a complete recovery of the normal phenotype for 100% of slightly *mantled* individuals and for 50% of severely *mantled* ones after 9 years in the field (Rival *et al.*, 1998b, 2000). Although the *mantled* abnormality is strongly associated with tissue culture, only a weak non-Mendelian transmission occurs via seeds (Rao and Donough, 1990). Alterations in genomic DNA structure that could be linked with the *mantled* phenotype have not been identified (Rival *et al.*, 1998c). Flow cytometric analyses have demonstrated a uniform 2C ploidy level (Rival *et al.*, 1997c) in normal and variant plant material. The *in vitro* cycle can affect the level of DNA methylation and can result in phenotypic alterations, i.e. somaclonal variation (Larkin and Scowcroft, 1981). Changes in DNA methylation on deoxycytidine (dC) residues are involved in the regulation of gene expression at the transcriptional level (Finnegan *et al.*, 2000), particularly during differentiation/dedifferentiation and as a response to environmental stresses (Brown, 1989; Phillips *et al.*, 1994; Oakeley *et al.*, 1997; Finnegan *et al.*, 1998; Demeulemeester *et al.*, 1999). Somaclonal variation may not be the result of a single causal mechanism (Finnegan *et al.*, 1993; Kaeppler *et al.*, 2000).

The occurrence of significant genomic hypomethylation in abnormal calluses (−4.5%; $P < 10^{-5}$) and leaves (−1.2%; $P < 10^{-5}$) from *mantled* regenerants has been demonstrated compared with their normal counterparts. The same patterns were observed in immature inflorescences (Jaligot *et al.*, 2000). Global DNA hypomethylation associated with local genetic or epigenetic defects has been documented in several cases of developmental abnormalities (Finnegan *et al.*, 1996, 1998; Nakano *et al.*, 2000).

Those sequences which, when misregulated, could potentially account for the *mantled* phenotype or could be used as markers for early detection of DNA methylation perturbation in the regeneration process have been investigated, i.e. methylation-sensitive RFLP and AFLP studies involving isoschizomeric enzymes *MspI* and *HpaII*.

RFLPs were used primarily to screen a pool of oil palm cDNA clones (Table 4.2.3) for methylation-dependent polymorphism (Jaligot *et al.*, 2002), while methylation-sensitive amplified polymorphism (MSAP) was used to generate a large number of relevant markers, exhibiting a differential methylation pattern depending on the normal/*mantled* phenotype but independent of the genetic origin of clones (Jaligot *et al.*, 2003). Possible

links between the observed hypomethylation and chromatin rearrangement were also examined, as DNA methylation often parallels the compaction of a genomic domain (Van Blockland *et al.*, 1997; Callebaut *et al.*, 1999; Wade *et al.*, 1999). By isolating oil palm relatives of the *Arabidopsis thaliana* MET1 DNA-methyltransferase gene, we may be able to determine how the *mantled* abnormality is generated, dysfunctions of genes of this

Table 4.2.3. Characterization of Southern blot polymorphism revealed by cDNA probes on isoschizomeric methylation-sensitive RFLP digestions of DNA extracted from oil palm embryogenic calluses.

Probe	LMC 458				LMC 464			
	Enzyme-dependent polymorphism (C ^m CGG)		Type-dependent polymorphism between <i>Hpa</i> II digestions (C ^m CGG)	Type-dependent polymorphism between <i>Msp</i> I digestions (C ^m CGG)	Enzyme-dependent polymorphism (C ^m CGG)		Type-dependent polymorphism between <i>Hpa</i> II digestions (C ^m CGG)	Type-dependent polymorphism between <i>Msp</i> I digestions (C ^m CGG)
	NCC	FGC			NCC	FGC		
CPHO 1	+	+	—	—	+	+	—	—
CPHO 5	+	+	—	—	+	+	—	—
CPHO 6	—	—	—	—	—	—	—	—
CPHO 7	+	+	+	—	+	+	—	—
CPHO 12	—	—	—	—	—	—	—	—
CPHO 17	+	+	—	—	+	+	—	—
CPHO 23	+	+	—	—	+	+	—	—
CPHO 27	+	+	—	—	+	+	—	—
CPHO 30	—	—	—	—	—	—	—	—
CPHO 31	+	+	—	—	+	+	—	—
CPHO 32	+	+	—	—	+	+	—	—
CPHO 34	+	+	—	—	+	+	—	—
CPHO 39	+	+	—	—	+	+	—	—
CPHO 42	+	+	—	—	+	+	—	—
CPHO 45	—	—	—	—	—	—	—	—
CPHO 48	+	+	—	—	+	+	+	—
CPHO 49	+	+	+	—	+	+	+	—
CPHO 53	+	+	+/-	—	+	+	+/-	—
CPHO 54	+	+	—	—	+	+	+	—
CPHO 59	—	—	—	—	—	—	—	—
CPHO 60	+	+	—	—	+	+	—	—
CPHO 62	+	+	+	+	+	+	+	+
CPHO 63	+	+	—	+	+	+	—	+
CPHO 64	+	+	—	—	+	+	—	—
CPHO 66	+	+	—	—	+	+	—	—
CPHO 69	+	+	—	—	+	+	—	—
CPHO 70	+	+	—	—	+	+	—	—

Plus (+) and minus (—) signs refer respectively to the presence or to the absence of detection of a category of differential banding pattern with a given probe. Non-reproducible patterns are marked (+/-).

family having been found to be linked to a number of developmental abnormalities.

The *mantled* abnormality involves a characteristic homoeotic modification of oil palm floral architecture, which implies that the activity of a specific subset of genes has been altered, at least within the flower and fruit tissues. Floral homoeotic genes of the 'MADS box' transcription factor family might be affected by the chain of events resulting in the *mantled* abnormality. In this regard, it is possible that *mantled*-dependent gene expression patterns may also exist *in vitro*. The identification of fingerprint genes displaying *mantled*-related expression at pre-planting stages could provide the means to formulate molecular markers for clonal fidelity. Studies have also been initiated in order to characterize variations in the abundance of individual polypeptides in relation to clonal conformity (Wilkins *et al.*, 1995).

3.2.2. Genetic transformation

Breeding objectives. The main goal for genetic engineering of oil palm is to alter oil quality by increasing oleic acid and lowering palmitic acid contents (Pryde, 1983; Cheah *et al.*, 1995; Kadir and Parveez, 2000).

Production of novel high-value products have also been targeted, including increased stearic acid, palmitoleic acid and ricinoleic acid contents, and producing biodegradable plastics (Parveez *et al.*, 1999). Shell thickness and resistance to fungal diseases and insect pests are other traits that can be manipulated to increase the yield and quality of the oil palm (Parveez, 1998).

The fatty acid biosynthetic pathway, which is common to all plants (Fig. 4.2.5), involves repeated incorporation of two-carbon units derived from malonyl-CoA to elongate the fatty acid chain to approx. 16 or 18 carbons (Stumpf, 1994). The reaction is initiated by acetyl-CoA. Malonyl-CoA is derived from acetyl-CoA by a carboxylase reaction involving acetyl-CoA carboxylase (ACCase). The main enzymes involved are collectively known as fatty acid synthase (FAS) and are localized in plastids and require a cofactor, ACP. The synthesized fatty acids are attached to ACP, which is removed by a specific thioesterase. The fatty acids move from plastids and are esterified to CoA. Further elongation and desaturation occur in the microsomes before the lipids either return to the plastids or are incorporated into triacylglycerols as storage oil in the microsomes. Regulation of enzymes can

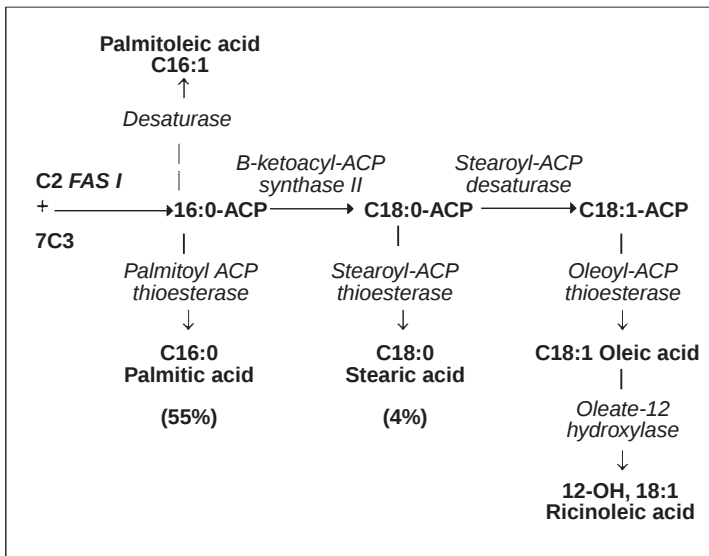


Fig. 4.2.5. Possible reactions involved in the modification of products of fatty acid synthetase.

vary in different crops as fatty acid contents are different (Sambanthamurthi *et al.*, 2000).

KAS II activity is rate-limiting in the mesocarp, resulting in the accumulation of palmitic acid. Increasing KAS II activity would increase stearoyl-ACP, which would subsequently be desaturated to oleic acid. The relationship between KAS II activity and the level of unsaturation has been studied in mesocarp of *E. guineensis* and *E. oleifera* as well as *E. guineensis* $\times$ *E. oleifera* hybrids, and there is a positive correlation between KAS II activity and both I.V and C18 unsaturated fatty acids (C18:1 + C18:2 + C18:3) (Sambanthamurthi *et al.*, 1996a). Furthermore, desaturase activity is not limiting as increased KAS II activity does not result in accumulation of stearic acid but an increase in unsaturated C18 fatty acids. The level of C16:0 is negatively correlated with the level of C18:1. This negative correlation confirms that palmitic acid accumulation is controlled by KAS II activity. Higher KAS II activity would cause more palmitic acid to be channelled to oleic acid.

In most plants, the major product of fatty acid biosynthesis in the plastids is oleic acid. A thioesterase highly active towards oleoyl-ACP has been described which ensures that oleic acid is released from ACP and exported out of the plastids. Plants that accumulate medium-chain fatty acids express a medium-chain-specific acyl-ACP thioesterase (Davies *et al.*, 1991; Pollard *et al.*, 1991). Crude oil palm mesocarp extract was assayed for thioesterase activity against different acyl-ACP substrates. Maximum activity was obtained with palmitoyl-ACP (Sambanthamurthi and Oo, 1990). Thus, palmitic acid accumulation in the mesocarp could be attributed to chain termination by the action of palmitoyl-ACP thioesterase. Mesocarp exhibits high thioesterase activity towards both palmitoyl-ACP and oleoyl-ACP. Thioesterases have been partially purified and shown to be two different proteins. Palmitoyl-ACP thioesterase activity could therefore be lowered independently of oleoyl-ACP thioesterase and hence reduce palmitic acid levels without reducing oleic acid levels. Oleic acid could be increased by increasing oleoyl-ACP thioesterase activity without increasing palmitoyl-ACP thioesterase activity.

KAS II and palmitoyl-ACP thioesterase are the main target enzymes for manipulation to produce high oleic acid at the expense of palmitic acid. Other enzymes that could contribute towards high oleic acid are stearoyl-ACP desaturase and oleoyl-ACP thioesterase. Appropriate temporal and spatial expression of the introduced gene is necessary. Thus, genetic engineering requires the isolation of mesocarp-specific regulatory sequences to ensure that the target genes are expressed in the mesocarp during oil synthesis.

Protocol. Oil palm transformation has been based upon biolistics, although stable transformation efficiency is low, i.e. 0.1–2.0% (Gordon-Kamm *et al.*, 1990; Bower and Birch, 1992). *Agrobacterium*-mediated transformation can be more efficient; however, the efficiency is still low and inconsistent (Parveez *et al.*, 2000). Transformation was initiated using five constructs carrying different promoters (Fig. 4.2.6): Emu (based on Adh1), maize Ubiquitin1, rice Actin1, cauliflower mosaic virus (CaMV) 35S and maize Adh1 (Chowdury *et al.*, 1997). Bombarded embryogenic cultures were exposed to 40 and 80 mg/l of BastaTM after 1 or 3 weeks. The presence of transgenes in resistant embryogenic cultures was confirmed by PCR and Southern analysis (Parveez and Christou, 1998). Transgenic plants were regenerated and their transgenic status verified by PCR, Southern hybridization and protein analysis by thin layer chromatography (Parveez, 1998, 2000). Transgenic plants > 3 years old were subjected to leaf painting and shown to be resistant to Basta (Fig. 4.2.7). Bombardment with six plasmid constructs, carrying different versions of GFP and driven by different promoters, into embryogenic cultures has also been successful. Transient expression of GFP in embryogenic cultures has been observed (Na'imatulapidah and Parveez, 2000; Parveez *et al.*, 2000). Immature embryos 11–12 WAA were transformed with pCAMBIA 1301 (*gusA* and *hpt* genes with the CaMV 35S promoter) (Roberts *et al.*, 1997) using the sonication-assisted *Agrobacterium*-mediated transformation (SAAT) method (Santarem *et al.*, 1998).

pPAsUbiSADN	UBI	BAR	NOS	UBI	P-AsDES	NOS
pPAs35SSADN	UBI	BAR	NOS	S5S	P-AsDES	NOS
pAsUbiSADN	UBI	BAR	NOS	UBI	FL-AsDES	NOS
pAs35SSADN	UBI	BAR	NOS	35S	FL-AsDES	NOS
PUbiSADN	UBI	BAR	NOS	UBI	FL-DES	NOS
p35SSADN	UBI	BAR	NOS	35S	FL-DES	NOS
pCB302-AT1	PNOS	BAR	NOS	352E	FL-AsTE	NOS
pCB302-AT2	PNOS	BAR	NOS	35Σ	FL-AsTE	NOS

Fig. 4.2.6. Plasmids used for transforming oil palm. 352E: CaMV 35S promoter with double enhancer; 35Σ: CaMV 35S promoter with a translational enhancer; Ubi: maize ubiquitin promoter; As: antisense; BAR: Basta resistance gene; TE: palmitoyl ACP thioesterase; KAS II: β-ketoacyl ACP synthase II; DES: Δ9-stearoyl-ACP desaturase; NOS: nopaline synthase poly-A terminator; PNOS: nopaline synthase promoter; P: partial length; FL: full-length.



Fig. 4.2.7. Transgenic oil palm plant 36 months old.

Two approaches were considered for producing higher oleic acid yields: (i) stimulation of KAS II activity by over-expressing the KAS II gene; and (ii) reducing thioesterase activity towards palmitoyl-ACP by introducing an antisense copy of the palmitoyl-ACP thioesterase gene (Parveez *et al.*, 1999). The goal of this project is to increase the oleate content in the mesocarp of oil palm, where the oil is being synthesized.

Accomplishments. Construction of the palmitoyl-ACP thioesterase, ketoacyl-ACP synthase II and Δ9-stearoyl-ACP desaturase gene transformation vectors driven by a mesocarp-specific promoter is in progress (Siti Nor Akmar *et al.*, 2001). Four transformation vectors have been constructed driven by constitutive promoters: pUbiSADN (full-length Δ9-stearoyl-ACP desaturase gene driven by maize ubiquitin 1 promoter),

p35SSADN (full-length 9-stearoyl-ACP desaturase gene driven by CaMV 35S promoter) and pCB302-AT1 and pCB302-AT2 (full-length antisense palmitoyl-ACP thioesterase gene driven by CaMV 35S promoter with different enhancers) (see Fig. 4.2.6). Transformation of the full-length antisense palmitoyl-ACP thioesterase and $\Delta 9$ -stearoyl-ACP desaturase gene constructs is in progress. Embryogenic and suspension cultures have been transformed with the constructs via co-bombardment, and transgenic embryogenic cultures are being selected for resistance to Basta.

Accumulation of stearic acid. Oil palm contains 9-stearoyl-ACP desaturase which desaturates stearoyl-ACP into oleoyl-ACP and finally into oleic acid (see Fig. 4.2.5). Introducing an antisense copy of the 9-stearoyl-ACP desaturase gene should cause stearic acid to be accumulated by reducing conversion of stearate to oleate (Knutzon *et al.*, 1992a,b). The expression of stearoyl-ACP desaturase gene driven by a seed-specific promoter increases the stearate content from 1.8% by weight (normal) to 39.8% in the seed of transgenic rapeseed plants with a concomitant reduction of oleate.

Four transformation vectors have been constructed for the production of high stearate transgenic oil palm: pPAsUbiSADN (partial-length antisense) 9-stearoyl-ACP desaturase gene driven by maize ubiquitin 1 promoter, pPAs35SSADN (partial-length antisense 9-stearoyl-ACP desaturase gene driven by CaMV 35S promoter), pAsUbiSADN (full-length antisense $\Delta 9$ -stearoyl-ACP desaturase gene driven by maize ubiquitin 1 promoter) and pAs35SSADN (full-length antisense 9-stearoyl-ACP desaturase gene driven by CaMV 35S promoter) (see Fig. 4.2.6). Transformation of the two partial-length antisense $\Delta 9$ -stearoyl-ACP desaturase gene constructs is in progress. Embryogenic cultures have been transformed with the constructs and some Basta-resistant cultures have been produced. Regeneration of resistant embryogenic cultures has been initiated. Construction of a full-length antisense $\Delta 9$ -stearoyl-ACP desaturase gene transformation vector driven

by the mesocarp-specific promoter is in progress (Siti Nor Akmar *et al.*, 2001).

Palmitoleic acid is produced by desaturation of palmitic acid. $\Delta 9$ -stearoyl-ACP desaturase, which acts mainly on stearic acid but could also use palmitic acid as a substrate to produce palmitoleic acid (see Fig. 4.2.5). Therefore, increasing stearoyl-ACP desaturase activity may result in the accumulation of palmitoleic acid, which has important pharmaceutical applications (Parveez *et al.*, 1999). Oil palm protoplasts can synthesize up to 30% of palmitoleic acid in the total lipids (Sambanthamurthi *et al.*, 1996b). Construction of two vectors driven by constitutive promoters, pUbiSADN (full-length) 9-stearoyl-ACP desaturase gene (driven by maize ubiquitin 1 promoter), and p35SSADN (full-length) 9-stearoyl-ACP desaturase gene (driven by CaMV 35S promoter), has been achieved. Embryogenic and suspension cultures have been transformed and some Basta-resistant cultures have been produced (Siti Nor Akmar *et al.*, 2001).

Ricinoleic acid synthesis. Oleic acid is converted to ricinoleic acid by the enzymatic action of oleate 12-hydroxylase (see Fig. 4.2.5). Introduction of the oleate 12-hydroxylase gene into oil palm could result in the synthesis of ricinoleic acid, which is important for the cosmetics and pharmaceutical industries and as polymers and high-grade lubricants (Parveez *et al.*, 1999).

Biodegradable thermoplastics. In bacteria, polyhydroxybutyrate (PHB) is derived from acetyl-CoA by a sequence of three enzymatic reactions. The first enzyme of the pathway, 3-ketothiolase, catalyses the reversible condensation of two acetyl-CoA moieties to form acetoacetyl-CoA. Acetoacetyl-CoA reductase subsequently reduces acetoacetyl-CoA to D-(-)-3-hydroxybutyryl-CoA, which is then polymerized by the action of PHB synthase to form PHB (Anderson and Dawes, 1990). Introduction of these genes into oil palm could result in the accumulation of PHB (Poirier *et al.*, 1992; Nawrah *et al.*, 1994; Mitsky *et al.*, 1997). Both PHB and related polyhydroxyalkanoates (PHA) are renewable sources of biodegradable thermo-

plastic materials. All three genes involved in PHB synthesis are to be driven by a different promoter. A second construct in which *phbA* gene is replaced with *bktB* gene would result in accumulation of PHBV rather than PHB (Masani *et al.*, 2000). The plastid-targeting sequence from the oil palm ACP gene (Rasid *et al.*, 1999) is being used to make the chimeric gene constructs because studies in *Arabidopsis* suggested that higher yield can be obtained when PHB production is confined to the plastids (Nawrah *et al.*, 1994). Both constructs are in a binary vector, flanked by matrix attachment regions of tobacco and using maize ubiquitin, CaMV 35S and rice actin promoters. Selection of Basta-resistant embryogenic cultures has been initiated (Siti Nor Akmar *et al.*, 2001).

3.3. Cryopreservation

Development of a cryopreservation protocol for oil palm somatic embryos is essential due to the large number of clones produced. A cryopreservation protocol was developed (Engelmann *et al.*, 1985); however, this technique was restricted to finger-shaped embryos. Improvements to the process involved an embryo dehydration step using silica gel, following a pre-treatment on high sucrose medium, before freezing in liquid nitrogen (Dumet *et al.*, 1993a). The 7-day pre-growth period previously used was completed by an additional dehydration period carried out either by placing the embryos in the air current of the laminar flow cabinet or in an airtight box containing silica gel. This approach allowed the freezing of standard embryos, thus suppressing the limitation imposed by the low production of finger-shaped embryos; moreover, proliferation recovery after thawing was generally higher and more rapid, reaching up to 100% in some cases. This process was successfully applied to 39 different oil palm clonal lines (Dumet *et al.*, 1993b). The effect of various sugars and polyols on the tolerance to desiccation and freezing of oil palm embryonic cultures was subsequently investigated (Dumet *et al.*, 1994). When embryos were cryopreserved after a desiccation period, survival was optimal with

fructose, galactose, sucrose and glucose, intermediate with maltose and lower with other compounds. The recovery of proliferation was best with embryos conditioned with sucrose.

Routine application of cryopreservation to oil palm somatic embryos is now standard. Cryopreservation of embryogenic lines is feasible (Chabrillange *et al.*, 2000), and can be utilized to reduce the loss of embryogenic capacity in suspensions during maintenance.

4. Conclusions

A range of biotechnological approaches, from somatic embryogenesis to biomolecular research, play an increasingly important role in breeding strategies for oil palm. They are fully integrated into programmes aimed at development, multiplication and dissemination of genetically improved material to the planters. Biotechnological approaches applied to oil palm breeding are having a major impact on micropropagation, marker-assisted breeding and, more generally, in physiological and molecular studies of the expression of genes of paramount agronomic value (flowering, abscission, disease resistance, oil quality, etc.).

Recent results in somatic embryo development from embryogenic suspensions open the way for the use of strategies based on the artificial seeds concept. Recent studies on DNA methylation have provided a first glimpse of the molecular changes associated with the *mantled* abnormality and it is consistent with the epigenetic characters observed, including reversion. We are now carrying out methylation-sensitive RFLP and AFLP studies, involving the isoschizomeric enzymes *MspI* and *HpaII*, in order to identify relevant markers, exhibiting a differential methylation pattern depending on the normal/*mantled* phenotype but independent of the genetic origin of clones. In parallel, we have examined a possible link between hypomethylation and chromatin rearrangement. We hope that by isolating oil palm relatives of the *A. thaliana* *MET1* DNA-methyltransferase gene, we will obtain useful information to explain how the *mantled* abnormality is generated.

Molecular marker data will be associated with selected phenotypic characters to define and apply MAS. Such use of molecular markers will increase the efficiency of conventional breeding in terms of accuracy and time. It is an early selection tool.

Transgenic oil palm has been successfully produced using the biolistic method. MPOB's primary goal for genetic engineering is to increase oleic acid content. An experimental transformation system using embryogenic suspensions has recently been established at CIRAD/Institut de Recherche pour le Développement (IRD) to understand gene expression related to the molecular phenomena underlying the *mantled* abnormality.

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4.3 *Phoenix dactylifera* Date Palm

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1. Introduction

1.1 Botany and history

The date palm *Phoenix dactylifera* L. is a long-lived dioecious species. It has a non-branching trunk, ending in a clump of leaves. The plant has a single terminal shoot apex, which ensures the growth lengthwise. Each leaf has an axillary bud, which may be vegetative, floral or intermediate (Bouguedoura *et al.*, 1990; Bouguedoura, 1991). Axillary buds can form offshoots or suckers during the juvenile phase of the palm that give rise to inflorescences during the mature phase. Inflorescences are formed from axillary buds at the top of the tree. Male and female flowers are borne by separate trees. Pollination is usually carried out by wind; however, hand pollination of pistillate trees is usual and required to obtain good fruit set. The root system is highly developed. The date palm was named with reference to the fruit shape, which resembles fingers (Latin: *dactylus*).

The first domesticated date palms are thought to have originated in the southern Near East (Zohary and Spiegel Roy, 1975). There are many other *Phoenix* species that have probably hybridized with the originally domesticated variety and have given rise to current cultivars. The earliest cultivation of

date palm dates from 3700 BC (Munier, 1973) in the region between the Euphrates and the Nile Rivers. Its current distribution to other areas of the globe has been determined by the climate requirements of the plant, between 9° and 39° latitude north and south of the equator (Munier, 1973), especially in dry and semi-arid regions.

1.2. Importance

The date palm has great socio-economic importance, especially in North Africa and the Middle East. In addition to its valuable fruit, the tree is cultivated for fuel, fibre and as shelter for ground crops. In areas of its culture, which include arid and semi-arid areas, the date palm contributes to the creation of a microclimate that enables agricultural development of other species. World production of dates is approximately 5,353,090 t (FAOSTAT, 2004). The leading producer countries are Egypt (1,115,000 t), Iran (875,000 t), Saudi Arabia (830,000 t), United Arab Emirates (760,000 t), Pakistan (650,000 t) and Iraq (400,000 t). Although most dates are consumed locally, there is a large export market (512,829 t); India, the United Arab Emirates, Pakistan and France are the leading importers of fruit. Iran and the United Arab Emirates are the leading exporters.

1.3. Breeding and genetics

The species can be propagated by offshoots and from seeds. Offshoots develop slowly and a select tree can develop zero to three offshoots per year and not more than 10–40 during its lifetime depending on the cultivar and the environmental conditions. Seed germination is easy but seedlings may require up to 10 years before flowering and fruiting. When plants are raised from seeds, the sex type of seedlings segregates approx. 50% males to 50% females. Seedling populations are heterogeneous. Nevertheless, sexual reproduction gives rise to new genotypes and provides the basis for selection of elite trees.

Nemec (1910) reported that the chromosome number was $2n = 2x = 28$ in the young developing embryos of a non-specified cultivar. Other reports have shown different chromosome numbers, e.g. $2n = 2x = 36$ (Beal, 1937), $2n = 2x = 32$ or $2n = 2x = 36$ (Al Salih and Al Rawi, 1987; Al Salih *et al.*, 1987; Ibrahim *et al.*, 1998). Recently a chromosome number of $2n = 2x = 26$ was observed in two Moroccan date palm cultivars and in their *in vitro*-regenerated plantlets (Loutfi, 1999). Such investigations are hampered by the absence of soft tissues in mitosis, especially from adult trees, and by the numerous and small chromosomes. A cytological method based on chromocyanin staining has been described (Siljak-Yakovlev *et al.*, 1996) and indicates the presence of sexual chromosomes carrying distinctive nucleolar heterochromatin.

1.3.1. Breeding objectives

The earliest breeding attempts were initiated about 50 years ago in the USA, Algeria and Morocco. In the USA, research objectives aimed to: (i) obtain new stable cultivars possessing parental characteristics by several back-crosses; and (ii) improve agronomic criteria of some cultivars by intervarietal hybridizations (Nixon and Furr, 1965; Ream and Carpenter, 1975). The programme produced several hybrid females and a series of back-crossed male lines possessing metaxenic effects. In Algeria the breeding programmes were conducted in order to create new geno-

types more adapted to the Saharan climate and with resistance to *bayoud* disease. In Morocco such programmes were focused on obtaining genotypes with high fruit quality and resistance to *bayoud* disease (Saaïdi, 1979). Thirty-eight female hybrids of good date quality have been obtained at this time; however, only two of them are resistant to *bayoud* disease and are recommended for large-scale propagation by *in vitro* culture (Anon., 1998). These projects did not achieve the expected results (Bouguedoura, 1991) due to the dioecious nature of the plants (i.e. there is no self-fertilization) and the slow rate of growth (the first flowering occurs after at least 5 years of vegetative growth). Traditional breeding of date palm is hampered by the long generation cycle of trees. Three controlled back-crosses may require > 30 years and obviously it takes several more years to obtain enough offshoots from the new selections for further testing under field conditions.

The date palm is attacked by different pests and diseases (Carpenter, 1966; Djerbi, 1988) caused by many groups of fungi and insects. The main diseases caused by fungi include the following: (i) *bayoud* (*Fusarium oxysporum* f. sp. *albedinis*), which enters vascular tissues, spreads to all parts of the plant and leads to death; (ii) *khamedj* (*Mauginiella scaetiae* Cav.), which affects the inflorescences and significantly reduces date yield; (iii) black scorch or *medjnoun* (*Ceratocystis paradoxa* (Dade) C. Moreau), which affects different organs of the date palm, especially the shoot tip; and (iv) *belaat* (*Phytophthora* sp.), which causes shoot apex rot. Date and coconut palms are also hosts of a phytoplasma that causes lethal yellowing.

The most devastating of all date palm problems is the vascular fusariosis caused by the imperfect fungus, *F. oxysporum* f. sp. *albedinis* (Malençon, 1934; Louvet and Toutain, 1973). This disease originated in date palm groves of southern Morocco, and has spread to other areas of North Africa, especially Morocco and Algeria, where > 12 million date palm trees have been destroyed. Some date palm cultivars are resistant to *bayoud* disease; however, these cultivars all bear dates of poor quality. Date palm breeding programmes have been established in Morocco (Louvet

and Toutain, 1973; Saaïdi, 1979, 1992) and Algeria (Fernandez *et al.*, 1995) to address the problem of *bayoud*. The ultimate goal of these projects has been to evaluate and select new genotypes with resistance to *bayoud* disease and with good fruit quality. Two approaches have been adopted: (i) the selection of elite cultivars from wild genotypes in date palm groves; and (ii) the controlled hybridization of females with superior quality dates with *bayoud*-resistant males (or *bayoud*-resistant females with males known for their good effect on fruit set) (Saaïdi, 1979).

Pests that attack the crop include: *Batrachedra amydraula* Meyer; *Asterolecanium phoenicis* Rao and *Ephestia figulilla* (Kehat *et al.*, 1974). Other species cause great damage. Foremost among them is *Parlatoria blanchardi* Targ., which was responsible for major losses of date production in North Africa in the past, before the development of an integrated control approach based upon biological control using the natural enemies of this pest such as the ladybird. *Ectomyelois ceratoniae* Zeller attacks date palm fruits in tropical areas. *Rhynchophorus ferrugineus* Oliv. (red palm weevil), which usually attacks coconut palm, has recently been observed in date palm trees in Pakistan, India and the Arabian Gulf countries. It is becoming the most destructive pest of date palm in the Middle East (Abraham *et al.*, 1998). The pest bores into the leaf bases at the top of the trunk, causing the entire crown to wither and the death of the plant.

Other date palm disorders are caused by environmental conditions, i.e. cold, drought or wet weather, e.g. blacknose, which appears in the presence of high humidity during the fruit ripening period. A problem related to an anatomical defect of floral axes (the crosscuts) has been observed with some varieties. Some other disorders of unknown cause are observed for the crop (Djerbi, 1988).

2. Molecular Genetics

2.1. Molecular markers

Date palm is a much-neglected plant with respect to molecular genetics and yet breeders realize that developments in this field are

critical for improving this important species. For example, there is no sure molecular method for distinguishing the date producing female trees from the male trees before the first flowering, which can occur > 5 years after planting. Furthermore, it is difficult to identify female cultivars according to their morphological characteristics outside fruiting time. As a result, the approximately 5000 named date palm cultivars (Anon., 1914) must be distinguished solely on the basis of their fruit characteristics.

These problems, together with the devastation caused by *bayoud* disease, require efficient early biochemical and/or molecular markers for date palm quality, resistance to *bayoud* disease and sex determination. Biochemical markers (Ziouti *et al.*, 1996) and anti-fungal components in root extracts (Assef *et al.*, 1986; Assef, 1987) that are related to the sensitivity or resistance of date palm to *bayoud* are being investigated.

2.1.1. Protein markers

Many protein (isoenzyme) systems have been screened in order to characterize date palm cultivars (Torres and Tisserat, 1980; Stegemann *et al.*, 1987; Baaziz and Saaïdi, 1988; Chandra-Sekhar and De Mason, 1988; Bennaceur *et al.*, 1991; Bendiab *et al.*, 1993). Some studies have involved the use of either fruit (Stegemann *et al.*, 1987) or seeds (Chandra-Sekhar and De Mason, 1988), but most studies have utilized leaf material sampled from seedlings of known parents (Torres and Tisserat, 1980; Bendiab *et al.*, 1993) or of adult plants (Baaziz and Saaïdi, 1988; Bennaceur *et al.*, 1991). Using 31 cultivars, Bennaceur *et al.* (1991) demonstrated that an identification key based upon five enzymes – glutamate oxaloacetate transaminase, endopeptidase (ENP), diapharase, phosphoglucomutase and leucine amino peptidase – is able to characterize 65% of the cultivars. Bendiab *et al.* (1993) analysed F₁ hybrid populations resulting from crosses of female *bayoud*-resistant and low-quality cultivars with a *bayoud*-resistant male or crosses of *bayoud*-susceptible and good-quality cultivars with a susceptible male for esterase, glutamate oxaloacetate transaminase, ENP and alcohol

dehydrogenase polymorphisms. They observed that in almost every case the most electrophoretic phenotypes obtained for each enzyme in these populations were similar to those of corresponding parent cultivars. They also found that F_1 populations were characterized by a higher electrophoretic polymorphism than the second crosses.

2.1.2. DNA markers

Methods using restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) technologies have been developed for date palm. Ait Chitt *et al.* (1993) described an efficient protocol for total DNA extraction from date palm leaves. Identification and characterization of two mini circular plasmid-like structures – an S and an R plasmid isolated from mitochondria of a date palm variety – were reported (Benslimane *et al.*, 1994; Benslimane, 1995; Bouachrine, 1997). Recently, 36 date palm varieties were studied using a polymerase chain reaction (PCR)-based approach; the mitochondrial genome of the *bayoud*-susceptible cultivars contains a plasmid-like structure, the S plasmid, whereas the *bayoud*-resistant varieties contain an R plasmid (Ouenzar *et al.*, 2001). Molecular markers involving RFLP have been evaluated for clone identification (Ait Chitt *et al.*, 1995; Cornicquel and Mercier, 1997). RAPD markers have also been described for several varieties from Morocco (Sedra *et al.*, 1998) and Tunisia (Benabdallah *et al.*, 2000; Trifi *et al.*, 2000). Several primers that show polymorphism with high reproducibility were selected; RAPD markers provided evidence of divergence between date palm varieties (Trifi *et al.*, 2000). In general, morphologically similar varieties, especially with respect to their date quality, were closely associated. Introduced varieties did not appear to be distinct from indigenous ones (Sedra *et al.*, 1998; Trifi *et al.*, 2000).

3. Micropropagation

Because of limitations of traditional date palm propagation methods, i.e. limited number of offshoots and difficulties in their

transplantation and growth, extensive efforts have been made to develop *in vitro* techniques for mass cloning. Mesbah (1985) attempted to micrograft shoots on to seedling rootstocks, but callus proliferation did not occur and there was no vascular connection between the rootstocks and the scions.

Date palm micropropagation by shoot tip culture is impractical, because of the absence of secondary meristems in this species and because the excised tissues rapidly turn brown and die. Most studies have focused on multiplication and rooting of shoot tips and lateral buds; however, it is difficult to demonstrate the origin of *in vitro* plantlets. A shoot tip technique was developed in order to obtain rooted shoots from seedlings, adult trees and offshoots (Zaid and Tisserat, 1983). Explants consisted of the apical dome with two or four leaf primordia and varied in size from 0.5 to 1 mm³. Such a technique has not been exploited for propagating date palm since it is nearly impossible to obtain a sufficiently large number of shoot tips from adult trees, and their proliferation *in vitro* has been very difficult to achieve.

4. Somatic Cell Genetics

4.1. Regeneration

Schroeder (1970) and Reuveni and Lilien-Kipnis (1974) conducted pioneer *in vitro* studies with date palm. In addition to some callus formation and root induction from the callus, the authors described the serious problem of browning of the plant growth medium and explants, which resulted in death of the material. Explants contain a high level of caffeoylshikimic acids (ranging from 190 to 430 µg/g fresh weight (FW) depending on the cultivar), which are oxidized by polyphenoloxidases (Macheix *et al.*, 1990), and must be considered as a major cause of explant and callus deterioration. Activated charcoal and polyvinylpyrrolidone (PVP) significantly reduce the amount of toxic phenolic compounds in plant growth medium and modulate qualitatively and/or quantitatively phenolic accumulation. On

charcoal-containing plant growth medium, callus cultures accumulate monomeric and oligomeric flavanols (proanthocyanidins) and caffeoylshikimic acids, whereas on control and PVP media the browning potential is higher and calluses accumulate other phenolics, including sinapic, ferulic and flavonoid derivatives and a small amount of caffeoylshikimic acid in comparison with callus on medium containing charcoal. The high content of methoxy-substituted phenols, i.e. sinapic and ferulic derivatives, has been associated with cell wall rigidity and loss of cell division together with tissue browning (El Hadrami, 1995).

Both organogenesis and somatic embryogenesis are being used to produce large numbers of date palm plants on a commercial scale. Callus and morphogenic cultures of date palm have been induced from different explants, including zygotic embryos, roots (Eeuwens, 1978; Sharma *et al.*, 1980), young leaves (Sharma *et al.*, 1984), shoot tips (Zaid and Tisserat, 1983; Gabr and Tisserat, 1985), fragments of stems excised from seedlings (Eeuwens, 1978), bases of young leaves obtained from the hearts of offshoots (Beauchesne *et al.*, 1986; El Hadrami *et al.*, 1995; Sharon and Chandramati, 1998), fragments of young inflorescences (Drira and Benbadis, 1985; Bhaskaran and Smith, 1992; Loutfi and Chlyah, 1998) and indeterminate axillary buds (Bouguedoura *et al.*, 1990). The most commonly used explants consist of segments taken from the hearts of offshoots, which often contain axillary buds (Poulain *et al.*, 1979; Beauchesne *et al.*, 1986; Sharma *et al.*, 1986; El Hadrami *et al.*, 1995; Veramendi and Navarro, 1996). Floral segments have also been utilized frequently (Drira and Benbadis, 1985; Loutfi, 1989; Bhaskaran and Smith, 1992; Loutfi and Chlyah, 1998; Loutfi, 1999). Endophytic microbial contamination is a major problem, and consists primarily of *Bacillus* sp. This problem occurs throughout the Maghreb countries, particularly in cultures initiated from offshoot tissues (Bouguedoura, 1993). Antibiotics such as gentamycin are often used with uneven success (Cherkaoui, 1997).

4.1.1. Somatic embryogenesis

Two different, but complementary, regeneration pathways were described in France (Poulain *et al.* 1979) and in the USA (Reynolds and Murashige, 1979; Tisserat, 1979). Poulain *et al.* (1979) described the initiation of vegetative buds from the heart of date palm offshoots as the first step of an organogenic pathway for date palm micropropagation. Reynolds and Murashige (1979) and Tisserat (1979) described the induction of embryogenic cultures and the development of somatic embryos from these cultures, and utilized various explants. Tisserat (1979) used plant growth media supplemented with activated charcoal and high concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (450.5 μ M). This technique has been used effectively by various commercial laboratories for date palm micropropagation by somatic embryogenesis.

Somatic embryogenesis of date palm has been confirmed by several groups (Zaid and Tisserat, 1983; Mater, 1986; Sharma *et al.*, 1986; Letouzé and Daguin, 1989; Bhaskaran and Smith, 1992; El Hadrami *et al.*, 1995; Sharon and Chandramati, 1998; Loutfi, 1999; El Bellaj, 2000).

Induction. Several factors are critical for the induction of embryogenic cultures, including explant type, genotype and plant growth regulators. Somatic embryogenesis has been achieved using offshoots or flower buds as explants. Although different auxins have been tested for embryogenic culture induction, i.e. picloram, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), naphthaleneacetic acid (NAA) and naphthoxyphenoxyacetic acid (NOAA), the optimum results occur in the presence of 2,4 D. Early studies demonstrated that relatively high concentrations of 2,4-D (450.5–901 μ M) in the presence of activated charcoal are necessary; however, more recent studies have shown that embryogenic cultures can be induced on a medium with lower concentrations of 2,4-D. Tissue browning constitutes a serious problem for *in vitro* cultures of date palm. Activated charcoal has been used by several authors to limit tissue browning under induction conditions for

embryogenic cultures (Tisserat, 1979; Sharma *et al.*, 1984; Mater, 1986; Bhaskaran and Smith, 1992; El Hadrami *et al.*, 1995; Loutfi, 1999). Other antioxidants or adsorbents have also been used, e.g. PVP, citric acid and ascorbic acid (Poulain *et al.*, 1979; Beauchesne *et al.*, 1986).

The induction medium consists of semi-solid modified Murashige and Skoog (1962) (MS) medium containing de Fossard vitamins, 22.6 μM 2,4-D, 22.2 μM benzyladenine (BA) and 150 mg/l activated charcoal (El Hadrami *et al.*, 1995; El Bellaj, 2000). The explants are incubated in the dark for 4–6 months at 26°C with subculturing every 4–5 weeks on a fresh medium of the same composition until the development of embryogenic callus. Genotypic differences with respect to embryogenic potential have been observed in explants derived from offshoots as well as explants obtained from inflorescences (El Hadrami *et al.*, 1995; Loutfi, 1999; El Bellaj, 2000). The acquisition of embryogenic potential has been described with respect to biochemical and histological changes (Baaziz *et al.*, 1994; El Hadrami and Baaziz, 1995; El Hadrami *et al.*, 1995). Embryogenic calluses of 'Bou-Sthammi noire' and 'Bou Feggous' cultivars were characterized by several soluble proteins, high levels of soluble, ionically and covalently bound peroxidases (isoperoxidases of R_f 0.60) and no detectable polyphenoloxidases. Non-embryogenic calluses (white and rooting callus) were typified by soluble acidic isoperoxidases of high mobility (R_f 0.75) and anodic ionically wall-bound polyphenoloxidases.

Maintenance. Embryogenic cultures can be maintained on a semi-solid medium consisting of the same basic composition as the induction medium in which growth regulator concentrations are reduced to 0.45 μM 2,4-D and 2.2 μM BA in order to allow culture proliferation as well as somatic embryo formation (El Hadrami *et al.*, 1995). Embryogenic cultures have also been successfully maintained in a medium devoid of growth regulators (Sharma *et al.*, 1986; Bhaskaran and Smith, 1992) or on medium containing 2.7 μM NAA and 0.44 μM BA

(Loutfi, 1999). Embryogenic cultures have been utilized to establish rapidly proliferating embryogenic suspension cultures using liquid medium of the same composition as the induction medium but devoid of growth regulators (Letouzé and Daguin, 1989; Bhaskaran and Smith, 1992). Cell suspensions are initiated by inoculating 5 g FW of friable embryogenic culture into 100 ml of liquid medium dispensed in 250 ml flasks. The flasks are shaken on a rotary shaker (100 rpm) and maintained in a 15 h photoperiod at 27°C. Suspensions are subcultured at 30-day intervals. At each subculture, cell suspensions are filtered first through a sterile 5 mm mesh fabric and subsequently through a 50 μm mesh fabric. Somatic embryos < 5 mm are transferred to fresh liquid medium (1 g FW per 100 ml of liquid medium). Somatic embryos > 5 mm are transferred on to semi-solid medium of the same composition for development and plant regeneration (Letouzé and Daguin, 1989).

Development and maturation. In general, somatic embryo development and maturation occur on semi-solid maintenance medium (Tisserat, 1979; Sharma *et al.*, 1986; El Hadrami *et al.*, 1995; El Bellaj, 2000); however, for somatic embryo maturation on semi-solid medium, the preliminary culture in liquid medium devoid of sucrose for 2 weeks followed by culture on 3% sucrose medium appears to improve somatic embryo development (Veramendi and Navarro, 1996). The addition of 0.1 μM abscisic acid (ABA) can improve maturation of somatic embryos; somatic embryos accumulate more proteins and carbohydrate than the controls grown in the absence of ABA (El Bellaj, 2000). Developing somatic embryos are transferred to low light intensity (of 28 $\mu\text{mol}/\text{m}^2/\text{s}$) with a photoperiod of 15 or 16 h.

Germination and conversion. Mature somatic embryos can germinate on the same semi-solid basal medium used for maintenance and maturation; however, the addition of 0.54 μM NAA to the medium allows rapid germination together with well-formed regenerated plantlets (Mater, 1986).

Plants 10–15 cm long with two or three leaves, a distinct tap root and two or three roots have been successfully transferred to soil. Some factors are critical in plantlet survival *ex vitro*, including the initial size of the regenerated plantlets, environmental conditions of acclimatization, composition of soil and chemical treatments against plant diseases. Soil is a mix of peat and gravette or peat moss and vermiculite in equal proportions. During their first 2–3 weeks of development *ex vitro*, the plantlets must be incubated in high relative humidity and then gradually adapted to greenhouse conditions. Incubation temperatures vary between 25 and 30°C during the acclimatization period. Several months are required to harden regenerated plants, but plants are then capable of surviving transfer to field conditions. Thousands of date palm somatic embryo-derived plantlets have been produced at a commercial scale in specialized laboratories around the world. Many of them have flowered and have produced normal fruit.

4.1.2. Organogenesis

This regeneration pathway involves several steps: (i) meristem induction; (ii) shoot multiplication; (iii) shoot elongation; and (iv) acclimatization.

Induction. Organogenic cultures have been induced from the internal face of young leaf bases taken from offshoots (Beauchesne *et al.*, 1986; Beauchesne, 1988). Induction generally requires 4–6 months under dark conditions in order to reduce the accumulation of phenolic compounds and tissue browning and to stimulate cell division. Induction is mediated by the interaction of several factors, including culture medium composition, genotype and the period of offshoot collecting from mother plants. In general, plant growth media with a high auxin:cytokinin ratio are required for induction (Poulain *et al.*, 1979; Beauchesne *et al.*, 1986). The induction medium consists of a modified MS basal medium supplemented with different combinations of plant growth regulators. Good results have been obtained with: (i) 5.4 μM

NAA, 4.9 μM indolebutyric acid (IBA) and 5–27 μM NOAA; (ii) 0.5 μM 2-isopentenyladenine (2iP) (Poulain *et al.*, 1979); and (iii) 5.4 μM NAA, 5.7 μM indole-3-acetic acid (IAA), 5–27 μM NOAA and 0.5–14.8 μM 2iP (Beauchesne *et al.*, 1986). Under the same culture conditions, there is significant variation in the frequency of induction among cultivars. Beauchesne (1982) noted that each genotype or cultivar appears to require a specific culture medium. Groups of cultivars that behave similarly with respect to percentage of shoot and callus formation have been distinguished for floral explants (Loutfi and Chlyah, 1998). Explants from offshoots and inflorescences can develop roots much earlier than bud initiation, which can inhibit further caulogenesis (Anjarne and Zaid, 1993; Loutfi, 1999).

Shoot multiplication. Shoot multiplication for date palm cultures occurs on plant growth mediums in which the auxin/cytokinin ratio is > 1 , e.g. 10.5 μM NOAA, 5.4 μM NAA and 5.7 μM IAA:2.2 μM BA, 5 μM 2iP and 4.6–23 μM kinetin (Beauchesne *et al.*, 1986). Plant growth regulator ratios < 1 have also been used for the proliferation of cultures initiated from young inflorescences (Loutfi and Chlyah, 1998), i.e. 2.7 NAA, 4.4 μM (or 8.8 μM) BA and 5 μM 2iP. Cultures are incubated in the light at 180 $\mu\text{mol}/\text{m}^2/\text{s}$ with a 16 h photoperiod. The multiplication index is closely dependent on plant growth combinations and cultivars. Some cultivars are more responsive than others to shoot multiplication. On proliferation medium, the multiple shoots resemble rosettes. Hyperhydricity is often observed in these cultures, but factors that affect this physiological disorder have not been identified. Preliminary studies have indicated that high levels of ammonium nitrate enhance rapid growth and hyperhydricity of date palm cultures (Bougerfaoui and Zaid, 1993).

Shoot elongation. Shoot elongation involves the transfer of shoot buds to a plant growth medium with a high auxin:cytokinin ratio (Beauchesne *et al.*, 1986; Loutfi and Chlyah, 1998). Good results have been obtained with a combination of 5.4 μM NAA, 2.2 μM BA

and 2.3 μM kinetin or a combination of 10.8 μM NAA and 4.4 μM BA (or 5 μM 2iP). Gibberellic acid (GA_3) (3–9 μM) has been utilized for 15–20 days during the first or second subculture in the elongation medium in order to improve shoot elongation (Beauchesne *et al.*, 1986; Loutfi, 1989). But GA_3 treatments cause the formation of abnormal plantlets. No special medium is used for rooting because date palm shoots root easily during the elongation medium. Well-formed and vigorous plantlets are obtained if they are transferred to multiplication medium in which the sucrose level is increased to 100 g/l (Beauchesne *et al.*, 1986). Rooted shoots adapt well in the greenhouse and appear to be true to type in the field. Approximately 1–2 years are required to regenerate plantlets ready for transfer to the greenhouse by organogenesis irrespective of the explant, which limits the use of the technique for the mass propagation of the species.

4.1.3. Haploid recovery

Attempts to regenerate date palm from anther cultures have resulted in cell division and globular embryo formation from uninucleate microspores (Brochard, 1981). Cold treatment of male inflorescences at 4°C for 48 h and a combination of two auxins and a cytokinin seem to be effective (Bouguedoura, 1991). The author has tested several nutrient media and different growth regulator combinations. Promising results were obtained when anthers were cultured on semi-solid Nitsh (1969) medium supplemented with 100 mg/l glutamine, 30 g/l sucrose, 5.4 μM NAA, 4.5 μM 2,4-D and 0.9 μM kinetin in the dark at 28°C. Nodular calluses appear to be embryogenic, but the putatively haploid embryos have failed to develop beyond the globular stage.

Ovules have been cultured on the same medium described above. Two growth regulator combinations favoured callus initiation from ovules: (i) 5.4 μM NAA, 4.5 μM 2,4-D and 0.9 μM kinetin; and (ii) 5.7 μM IAA, 4.5 μM 2,4-D and 4.4 μM BA. Optimum callus formation occurred from flowers taken from closed spathes in which the embryo sacs contained undifferentiated cells. Carpels enlarged and became quite prominent, and

nodular and embryogenic calluses were induced from the embryo sacs. Somatic embryos developed from the latter cultures, whereas only roots developed from the former calluses. On medium supplemented with 4.9 μM IBA, various stages of somatic embryos developed, although plant recovery did not occur.

4.2. Genetic manipulation

4.2.1. Genetic transformation

There have been no reported studies on genetic transformation of date palm. The embryogenic and organogenic pathways are highly efficient, and studies in this area should be a high priority.

4.2.2. Mutation induction and somaclonal variation

The International Atomic Energy Agency (IAEA) has sponsored a regional Technical Cooperation Project in the Maghreb on the use of induced mutations to recover date palms with resistance to *bayoud* disease. The protocol involves: (i) isolation and characterization of the phytotoxin(s) from the *bayoud* fungus; (ii) establishment of embryogenic suspension cultures of date palm cultivars; (iii) mutation of embryogenic cultures; (iv) *in vitro* selection for resistance to the phytotoxin(s); (v) regeneration of plants from phytotoxin-resistant embryogenic cells; and (vi) verification of resistance of regenerated plants. Work is underway with respect to isolation and characterization of *Fusarium* phytotoxins and the control of all the different steps of date palm regeneration from suspension cultures.

Embryogenic cultures from leaf explants of young plants were irradiated (γ -rays) on semi-solid medium, and suspension cultures were established (Fki *et al.*, 2002). Morphologically normal plantlets were then regenerated and analysed for DNA content by flow cytometry. The authors reported that all regenerated plants were diploid with the same DNA (2C) content as the controls (initiated without callus irradiation).

4.3. Cryopreservation

Medium-term conservation of date palm tissue has been reported for *in vitro* shoot buds and callus cultures (Bekheet *et al.*, 2002). After 12 months of storage at 5°C in the dark, a relatively high percentage of cultures remained viable in a medium supplemented with sorbitol at 40 g/dm² as an osmotic agent. Genetic engineering and breeding studies with date palm that rely upon biotechnology will require a constant supply of morphogenic cultures representing different genetic characteristics (Tisserat *et al.*, 1982). Embryogenic date palm cultures have been cryogenically stored in liquid nitrogen at -196°C for several months (Tisserat, 1981). Embryogenic date palm cultures were immersed in cold half-strength cryoprotective mixture consisting of polyethylene glycerol, glucose and dimethyl sulphoxide for 5 to 10 min on ice. The material was centrifuged, supplemented with 1 ml full-strength cryoprotectant mixture and stored in a programmable freezer to -30°C. Subsequently they were transferred to liquid nitrogen (-196°C). After 3 months in liquid nitrogen, the cultures were thawed rapidly by swirling tubes in a 60°C water bath. The cryoprotective mixture was diluted by gradual addition of sucrose (3%) to avoid osmotic shock. Cultures were transferred to a semi-solid medium without hormones for somatic embryo development. Enzyme polymorphisms for five enzymes (alcohol dehydrogenase, esterase, peroxidase, phosphoglucumutase and phosphoglucoisomerase) were analysed in leaves of regenerated plantlets. Isosyme patterns were similar for regenerated plants derived from frozen and non-frozen cultures (Ulrich *et al.*, 1982). MyCock *et al.* (1997) also reported that late globular/early torpedo stage date palm embryos could be successfully cryopreserved. Cultures were pre-treated with a cryoprotectant mixture consisting of polyethyleneglycol, glucose and dimethyl-

sulphoxide and then dried to a water content of 0.4–0.7 g/g.

5. Conclusions

Date palm has been regenerated from zygotic embryo and elite selections via somatic embryogenesis and organogenesis. Although industrial production of date palm by these regeneration pathways is a reality, there has been little interest in the genetic modification of outstanding cultivars using these same techniques. In the Maghreb countries, where *bayoud* disease is a serious epidemic, *in vitro* genetic modification of elite cultivars for *bayoud* resistance could be the solution for this problem. Little is known about date palm molecular markers in relation to *bayoud* disease, early sex determination of young plants and cultivar identification. The establishment of a date palm genomic map is important; however, basic genetic studies to firmly establish the chromosome number of this crop are still missing. Studies related to date palm genetics, expansion of the crop gene bank and biodiversity management using molecular tools are important goals. At the same time, *in vitro* manipulation of cell and tissue cultures and transformation technologies are essential to improve existing cultivars.

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5

Bromeliaceae

The *Bromeliaceae* is a family of herbaceous, perennial plants confined to the tropics and subtropics of the New World, with the exception of a single species, *Pitcairnia felicianana* (A. Chev.) Harms & Mildbr., which was discovered in Guinea in West Africa. The *Bromeliaceae* contains about 58 genera and 1400 species and is divided into two distinct habitat groups, the terrestrial and the epiphytic (Watson and Dallwitz, 1992 onwards). Pineapple has a

terrestrial habitat but shows some features of the epiphytes: storage of small quantities of water in the axils of the rosette of stiff narrow leaves, the presence of special water storage tissues in the leaves and the ability to endure considerable periods of drought. Many bromeliads are grown for ornamental purposes, particularly as indoor plants. Pineapple, however, is grown for its edible fruit.

Reference

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5.1 *Ananas comosus* Pineapple

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1. Introduction

1.1. Botany and history

Pineapples are monocotyledons with a short stem containing a rosette of long narrow leaves that are often spiny. The leaves of some cultivars yield a strong white silky fibre used for making fabrics or cordage. Plants have a dense terminal inflorescence coalescing to produce a syncarp formed by the almost complete fusion of many fruitlets. In the edible cultivars, hundreds of parthenocarpic berry-like fruitlets are fused to form a multiple fruit which can be eaten fresh or processed as a canned, crystallized or juiced product. The fruit is surmounted by a crown of small leaves, which may be used for propagation. Suckers and slips that arise as vegetative branches from the stem or peduncle axils, respectively, may also be used for propagation. The pineapple root system is shallow and rather limited (Purselove, 1972).

Based on the key of Smith and Downs (1979), the genus *Ananas* contains seven species: *A. comosus*, *A. ananassoides*, *A. nanus*, *A. bracteatus*, *A. paraguayensis*, *A. fritzmuelleri* and *A. lucidus*. The closely related genus, *Pseudananas*, contains the monotypic *P. sagenarius*. This key, however, is based largely on quantitative traits, which are strongly influ-

enced by the environment and, in some cases, on qualitative traits such as leaf spiness, which are governed by only a few genes (Leal and Coppens d'Eeckenbrugge, 1996). Molecular studies suggest a revision of the current classification system is needed that would lead to fewer species within the genus *Ananas*.

Pineapple is native to South America and was first seen by Europeans when Columbus landed on the inhabited island that he named Guadeloupe on 4 November 1493 during his second voyage to the New World. Although primitive pineapples are still found in a wild state throughout Brazil as far south as Paraguay, the centre of origin is thought to be in the Orinoco and Rio Negro river basins of northern Brazil, Colombia, Venezuela and northern Argentina. These regions contain the greatest level of diversification within the species (Collins, 1948; Coppens d'Eeckenbrugge *et al.*, 1997).

The pineapple was closely associated with the indigenous peoples of the region and was cultivated and dispersed during their migrations. The pineapple was used not only as a fresh fruit, but also for wine making, twine and net making and medicinal purposes and the rotted fruit for poisoning the tip of arrows (Collins, 1948; Collins, 1951; Leal and Amaya, 1991; Leal and Coppens d'Eeckenbrugge, 1996). Crowns,

slips and suckers withstand considerable desiccation and resume growth when planted. Consequently pineapples are easy to establish in new areas and have spread rapidly throughout the tropics.

1.2. Importance

World production of pineapple is estimated to be > 14.6 million t annually (FAOSTAT, 2004; Malezieux, 2000). More than 70% is consumed locally in the area of production. Although only a third of its output is utilized for processing, pineapple products account for more than two-thirds of the trade in pineapple by value. The processing industry is dominated by a single cultivar, 'Smooth Cayenne', with export earnings estimated at US\$1.2 billion for countries in Asia and parts of Africa and Latin America.

1.3. Breeding and genetics

Most varieties of *A. comosus* are self-incompatible due to the inhibition of pollen tube growth in the upper third of the style (Kerns, 1932), which is gametophytically controlled by a single locus with multiple alleles (Brewbaker and Gorrez, 1967). Some cultivars exhibit partial incompatibility (Cabral *et al.*, 2000), which may be temperature-dependent. Self-compatibility is common in wild pineapples. The wild types, *A. ananassoides* and *P. sagenarius*, are either partially or completely self-compatible. Fertilization occurs usually within 2 h of pollination and the ovule will collapse within 7 h following anthesis if fertilization has not occurred (Rao and Wee, 1979).

All *A. comosus*, and in fact most species within the *Bromeliaceae*, have a diploid number of 50 small, spherical chromosomes ($2n = 2x = 50$) (Collins and Kerns, 1931; Marchant, 1967; Brown and Gilmartin, 1986; Brown *et al.*, 1997). Within *Ananas*, there are triploid, tetraploid and heteroploid cultivars (Capinin and Rotor, 1937; Collins, 1960). Triploids arise when unreduced egg gametes are fertilized by normal haploid pollen (Collins, 1933). *Pseudananas sagenarius* is a naturally

occurring tetraploid with 100 chromosomes (Collins, 1960). Triploids are generally more vigorous than diploids (Collins, 1933) and can be commercially acceptable (Leal and Coppens d'Eeckenbrugge, 1996), but tetraploids are generally inferior to triploids and diploids. They are slow to flower and produce small fruit with a lower sugar content (Kerns and Collins, 1947).

The percentage of ovules producing seed is low in *A. comosus* 'Cayenne' (4–11%) compared to other cultivars (*A. comosus* var. *bracteatus* 35%) (Chan *et al.*, 2003). Cleistogamy can occur in some *A. comosus* cultivars (Chan *et al.*, 2003).

It is generally recognized that the indigenous peoples of South America contributed substantially to the domestication of the pineapple (Leal and Coppens d'Eeckenbrugge, 1996), probably through the selection of spontaneous mutations expressing desirable traits. The types found growing in and around villages usually exhibit desirable traits, e.g. improved palatability, improved fruit size, seedlessness, smooth leaves and in some cases improved leaf fibre properties which are not commonly found in wild types (Collins, 1951; Coppens d'Eeckenbrugge *et al.*, 1997).

Pineapple is very heterozygous and improvement of many different characteristics is possible. The first major hybridization programme was at the privately funded Pineapple Research Institute (PRI) in Hawaii from 1914 to 1972. The objectives initially were to replace 'Smooth Cayenne' as a processing cultivar, but eventually expanded to include fresh fruit. Both intraspecific and interspecific crosses were conducted and selection encompassed many aspects of productivity, fruit quality and pest and disease resistance. The PRI programme developed cultivars with greater resistance to root and heart rot (*Phytophthora cinnamomi*), heart rot (*Phytophthora parasitica*), pink disease, fruit marbling, fruitlet core rot and with some tolerance of mealybug wilt disease and root knot and reniform nematodes. Resistance to the physiological disorder blackheart was also demonstrated as well as improvements in ascorbic acid content, yield, canned product quality, fibrosity and higher or lower

acidity (Dull, 1965; Rohrbach and Pfeiffer, 1975; Williams and Fleisch, 1993; Rohrbach and Schmidt, 1994). Clonal selection was also used in the PRI programme and 'Cayenne' selections representing up to 30 different mutations were obtained (Collins and Kerns, 1938).

Many pineapple-producing countries now conduct small- to medium-scale hybridization and selection programmes. Most breeding has utilized the different varieties of *A. comosus* such as 'Cayenne', 'Queen', 'Mordilona', 'Spanish' and 'Pernambuco' or hybrids thereof to produce segregating populations of seedlings for screening and selection. Objectives have been similar to those of the PRI, but are focused more on the fresh fruit market and often have included additional characteristics, e.g. resistance to fusariosis caused by *Fusarium moniliforme* var. *subglutinans* (Cabral *et al.*, 1993, 1996; Hidalgo *et al.*, 1998), reduced incidence of translucency (Leal and Coppens d'Eeckenbrugge, 1996) and a shorter cropping cycle (Chan and Lee, 2000).

2. Molecular Genetics

Despite the economic importance of the crop, very little is known about the molecular genetics of pineapple. No molecular markers have been used in breeding programmes to date, although they could be of tremendous use if they could be linked to important agronomic traits or to disease/pest resistance. Only recently have genes been isolated, described and utilized in genetic transformation programmes.

2.1. Gene cloning

Cazzonelli *et al.* (1998) cloned and characterized two pineapple genes coding for the enzymes 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase. These enzymes catalyse the last two steps in the biosynthesis of the plant hormone ethylene, and ultimately control the amount of the hormone produced by the plant. Ethylene is involved in a wide range of

developmental processes from seed germination to senescence (Zarembinski and Theologis, 1994). Ethylene is also a very important factor for regulating the ripening of climacteric fruits. Although pineapple fruits are considered to be non-climacteric, both ethylene biosynthetic genes are up-regulated in the flesh of pineapple fruits during ripening, raising questions about the role of this hormone in pineapple ripening (Cazzonelli *et al.*, 1998, 1999).

Flowering initiation in pineapple is possibly triggered by a burst of ethylene induced by environmental cues (cool nights followed by warm days). Flowering synchronization is an important agronomic practice since it allows subsequent synchronization of harvesting. Pineapple crops are artificially induced to flower by spraying with ethylene-releasing compounds such as ethephon; however, a significant proportion of plants experience early natural flower initiation and, as a result, fruit ripening is asynchronous. An ACC synthase gene, different from the one mentioned above, putatively involved in the initiation of flowering in pineapple, has been cloned (Botella *et al.*, 2000). Silencing of this particular gene could avoid natural flowering until artificial induction is performed. As a consequence, synchronization of fruiting and ripening would occur, allowing the development of mechanized harvesting. Transgenic pineapple plants containing genetic constructs to inactivate this gene as well as the ripening-related ACC synthase were produced in 1998 and are now being evaluated in field trials (E. Firoozabady, J.R. Botella and N. Gutterson, unpublished; Botella *et al.*, 2000).

Blackheart is a fruit defect arising from the exposure of pineapples to temperatures < 20°C. The internal pineapple flesh turns brown from the overproduction of phenolic substances. The incidence of blackheart can be negligible to quite serious depending on the temperatures and the duration fruit is stored after harvest or on environmental conditions during fruit development in the field. The enzymatic complexes responsible for the production of phenolics in plants have been extensively studied and it has been shown that polyphenol oxidase (PPO)

is responsible for browning defects in several plant species, including potatoes (Bachem *et al.*, 1994). Stewart *et al.* (2001) have cloned a PPO gene which is highly induced in conditions that produce blackheart in pineapple fruits. This gene has been targeted for silencing and transgenic plants are now being evaluated in the field.

Other genes that confer important agronomic traits are needed. Among the multiple possible targets, resistance to disease, insect and nematode attack will have the biggest effect on production. Genetic modifications leading to increased fruit nutritional quality and taste will appeal to consumers, helping to decrease the current scepticism towards genetically modified foods.

2.2. Marker-assisted selection

Genetic classification in the genus *Ananas* was originally based on quantitative, morphological (Smith and Downs, 1979) and qualitative variables. Such classifications were initially re-evaluated using enzymatic markers (isozyme analysis). In more recent years, several DNA-based marker techniques, such as random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) analysis, have been used. There are no reports of these techniques being used to directly assist hybridization strategies in pineapple.

2.2.1. Protein markers

Isozymes were used in the first systematic studies of the pineapple by Garcia (1989), with eight enzyme systems involving ten loci, and by Aradhya *et al.* (1994), with six systems involving seven loci. They found a high heterozygosity and polymorphism in *A. comosus*, with most variation between the botanical varieties. In some cases, clones from the same cultivar showed differences in some systems. DeWald *et al.* (1992) tested eight enzymatic systems and found variable, well-resolved banding patterns for peroxidase (PER) and phosphoglucumutase (PGM). Their study and that of Laempet and Saghuanrungsirikul (1998) showed incom-

plete correspondence between cultivars and zymotypes, and therefore were unreliable for cultivar identification. Pérez *et al.* (1995) applied the isozyme analysis technique to detect mutant lines developed by ^{60}Co gamma irradiation of callus, while Arias Valdes *et al.* (1998) used the technique to evaluate ploidy levels.

2.2.2. DNA markers

DNA-based markers are more sensitive than protein markers and are widely used for cultivar identification, for gene isolation and, perhaps most significantly, for monitoring the segregation of chromosome regions known to be involved in controlling agronomic characters (quantitative trait loci or QTLs). In pineapple this technique has been used mainly to classify cultivars.

Ruas *et al.* (1995) used RAPD analysis to estimate the relationships among four cultivars. Polymorphism was observed in agreement with classifications based on morphological characters. Noyer *et al.* (1996) used RFLP analysis on ribosomal RNA and found the genus *Ananas* to be very homogeneous. The results suggested that variation within *A. ananassoides* could have constituted the origin of *A. comosus*.

Duval *et al.* (2001) used RFLP markers to analyse the genetic diversity of the genus *Ananas*. A total of 294 accessions were studied using 25 polymorphic probes (markers). Factorial analysis of the results and species dispersion in the diversity tree failed to reveal clear species boundaries, even though some species appeared to be reasonably well grouped. A large number of accessions (168) were studied in the cultivated species, *A. comosus*, but, despite the morphological variation observed among the different varieties, RFLP analysis indicated that they are well grouped with a low level of molecular diversity. The analysis of a limited number of accessions from the related genus *Pseudananas* also failed to reveal clear differences, and the authors therefore suggested reclassification of the taxonomy by fusing both genera into one genus and two species. This study indicated that variability was generally continuous between current

species, and *A. comosus* and *A. ananassoides*, on the basis of the new classification, appear to be the most diverse groups.

In the long term, significant advances in mapping technologies and the study of whole genomes can be confidently predicted. A major European Union (EU)-funded project aims at characterization and gene mapping for linking markers to morphological traits and disease resistance (Coppens d'Eeckenbrugge *et al.*, 2000).

3. Micropropagation

The development of micropropagation for the rapid multiplication of cultivars has been stimulated by the need to develop rapid clonal propagation procedures and by the large and regular demand for planting material. Traditional methods of pineapple propagation usually produce up to ten plants per annum, using crowns, slips and suckers from a single plant. Sectioning of these components, including the stem, can deliver up to 100 plants. Chlorfurenol can be used to enhance slip production as much as 30-fold. According to Pannetier and Lanaud (1976), 1 million plants could theoretically be micropropagated in 2 years from a single pineapple axillary bud.

The earliest report of pineapple *in vitro* propagation (Aghion and Beauchesne, 1960) and many other early studies (Mapes, 1973; Mathews *et al.*, 1976; Drew, 1980) were concerned with culture establishment, multiple shoot formation and plant regeneration. Subsequent studies have concentrated on methods to optimize proliferation; pineapple micropropagation is currently being used commercially by the pineapple industry to rapidly multiply new cultivars for early release (Smith and Drew, 1990). In practice, micropropagation is used for the establishment of multiplication blocks, which then provide conventional planting material for larger production blocks. This is because micropropagated plants are expensive and there are grower concerns that genetic off-types (somaclonal variants) will be produced in large numbers. The initial limited use of micropropagation for multiplication of new

cultivars allows screening of variants with respect to fruit characteristics before conventional methods of multiplication are used.

Table 5.1.1 summarizes some of the methods developed for pineapple micropropagation. The most commonly used explant for initiating cultures is the axillary bud, which is dissected from crown leaves. Fitchet (1990) utilized the crowns of some cultivars, e.g. 'Smooth Cayenne', and suggested that they should first be desiccated for a short period to break bud dormancy. Although contamination of the explants is common, owing to the closely packed whorl of leaves in the crown, which traps water and airborne particles, enough buds can usually be obtained to ensure successful establishment of some explants following surface disinfection. Murashige and Skoog (1962) semi-solid medium (MS), supplemented with a cytokinin, usually benzyladenine (BA) at 9–22 μM , is commonly utilized. At these concentrations, a cultivar such as 'Smooth Cayenne' can produce ten to 15 plants per month. Multiplication can be enhanced two to three times by the use of agitated liquid medium (Mathews and Rangan, 1979; DeWald *et al.*, 1988; Moore *et al.*, 1992) and further refinements have been made that involve the use of temporary immersion systems, resulting in bud clusters from which a high number of shoots can be differentiated (Firoozabady *et al.*, 1995; Escalona *et al.*, 1998). A novel micropropagation method was developed by Kiss *et al.* (1995) that involved the initiation of etiolated shoots and their subsequent multiplication along nodal segments when placed horizontally on the culture medium.

After shoots have been produced and multiplied they are usually transferred to semi-solid MS medium containing an auxin, e.g. 3-indolebutyric acid (IBA) at 2.5–10 μM , or to hormone-free medium for root development. Plants can be successfully grown in a soil-less potting mix in a glasshouse or shade house prior to hardening off in full sun and eventual establishment in the field. The use of *Azobacter* or endomycorrhizal fungi has also been suggested to improve the growth of micropropagated plants (Gonzales *et al.*, 1996; Guillemin *et al.*, 1996; Matos *et al.*, 1996).

Table 5.1.1.1. Review of methods used for the micropropagation of pineapple.

Stage I	Stage II	Stage III	Stage IV	Reference
Explant: Axillary and terminal buds from crown Medium: Nitsch with 0.4 μ M BA and 0.5 μ M NAA	Medium: MS with 9 μ M kinetin, 10 μ M IBA and 10 μ M NAA 8 plantlets/month	Medium: MS with 0.5 μ M NAA and 2 μ M IBA	'Soil'	Mathews <i>et al.</i> (1976)
Explant: Axillary buds from slips and suckers Medium: MS, hormone-free	Medium: MS with 10 μ M BA and 10 μ M kinetin 50 plantlets/month (callus involved)	'Not stated'	'Gro-pots'	Drew (1980)
Explant: Axillary bud from crown Medium: MS with 25% coconut water	Medium: MS with 2–5 μ M BA 3 plantlets/month	Medium: $\frac{1}{2}$ MS, hormone-free	'Not stated'	Zepeda and Sagawa (1981)
Explant: Axillary bud from crown Medium: MS with 9 μ M BA and 10 μ M NAA	Medium: as for Stage I, but suspension cultures 17 plantlets/month for 'Smooth Cayenne'; 76 plantlets/month for 'Perolera'	No roots	'Commercial soil mix' Plantlets > 2.5 cm	DeWald <i>et al.</i> (1988) Moore <i>et al.</i> (1992)
Explant: Axillary bud from crown Medium: MS with 2 μ M BA and 1 μ M IAA	Medium: MS with 2 μ M BA 'Plantlets halved or quartered during subculture' 10 plantlets/month	Medium: MS, hormone-free	'Peat/perlite mix' Plantlets 2–3 cm	Cote <i>et al.</i> (1991)
Explant: Axillary bud from crown Medium: MT with 9 μ M kinetin, 10 μ M IBA and 10 μ M NAA	Medium: MT with 9 μ M kinetin and 10 μ M NAA 14 plantlets/month	Medium: MT with 5 μ M NAA and 500 mg/l malt extract	'Peat/perlite/sand'	Fitchet (1990) Fitchet-Purnell (1993)
Explant: Axillary bud from stem Medium: MS with 10 μ M BA and 3 μ M NAA	Medium: as for Stage I 8 plantlets/month	Medium: MS with 1.5 μ M IBA and 0.6 μ M IAA	'Not stated'	Osei-Kofi and Adachi (1993)
Explant: <i>In vitro</i> plantlets Medium: MS with 10 μ M NAA in the dark	Medium: N6 with 23 μ M kinetin and 20 μ M BA 'Etiolated shoots placed horizontally on medium' 60 plantlets/month	Medium: MS, hormone-free	'Soil' Plantlets 8 cm	Kiss <i>et al.</i> (1995)
Explant: Apical bud from crown Medium: MS with 4.5 μ M BA and 0.5 μ M NAA	Medium: as for Stage I, except 9 μ M BA 'Regeneration from proliferating callus at base of plantlets' 56 plantlets/month	Medium: MS with 10 μ M IBA	'Not stated'	Devi <i>et al.</i> (1997)

IAA, indoleacetic acid; IBA, indolebutyric acid; BA, benzyladenine; NAA, naphthaleneacetic acid; MS, Murashige and Skoog (1962); MT, Murashige and Tucker (1969); N6, Chu (1978); Nitsch, Nitsch (1951).

4. Somatic Cell Genetics

4.1. Regeneration

4.1.1. Somatic embryogenesis

Pineapple plants can be readily regenerated from cell or callus cultures. When the cytokinin is supplemented with an auxin such as naphthaleneacetic acid (NAA), the development of 'lumpy' tissue with protocorm-like bodies or callus can result (Mathews and Rangan, 1979, 1981; Wakasa, 1989; Devi *et al.*, 1997). Proliferation of these types of tissue often results in cultures with an enhanced regeneration capacity via either organogenesis (Fitchet, 1990) or somatic embryogenesis (Daquinta *et al.*, 1996; Cisneros *et al.*, 1998).

Daquinta *et al.* (1996) used the first three leaves from the apex to induce embryogenic cultures and found a close similarity between zygotic and somatic embryogenesis. The addition of 3,6-dichloroanisic acid (Dicamba) and BA to the culture medium increased the callus weight only slightly, but had a significant effect on the regeneration capacity.

4.1.2. Organogenesis

Mathews and Rangan (1981) induced organogenic callus on medium supplemented with NAA and coconut water, from which shoot bud primordia differentiated after subculture on medium containing coconut water and casein hydrolysate. The same medium was used by Smith *et al.* (2002) for regenerating transformed plants from callus cultures. Wakasa (1989) grew nodular bodies on media supplemented with 10 μ M NAA and 9 μ M or higher of BA and found that shoots differentiated on media containing the same plant growth hormones or only BA, but when BA was omitted from the media only roots were produced. Using Wakasa's (1989) protocol for 'Smooth Cayenne', Graham *et al.* (2000) initiated callus on excised leaf bases from well-developed micropropagated shoots within 3–4 weeks (Fig. 5.1.1). Callus was maintained on the same medium for proliferation, producing different types of callus, very similar to those described by Taylor *et al.* (1992) for sugarcane. The majority of calluses (organogenic) consisted of compact, hard, globular, yellow-green, smooth-surfaced

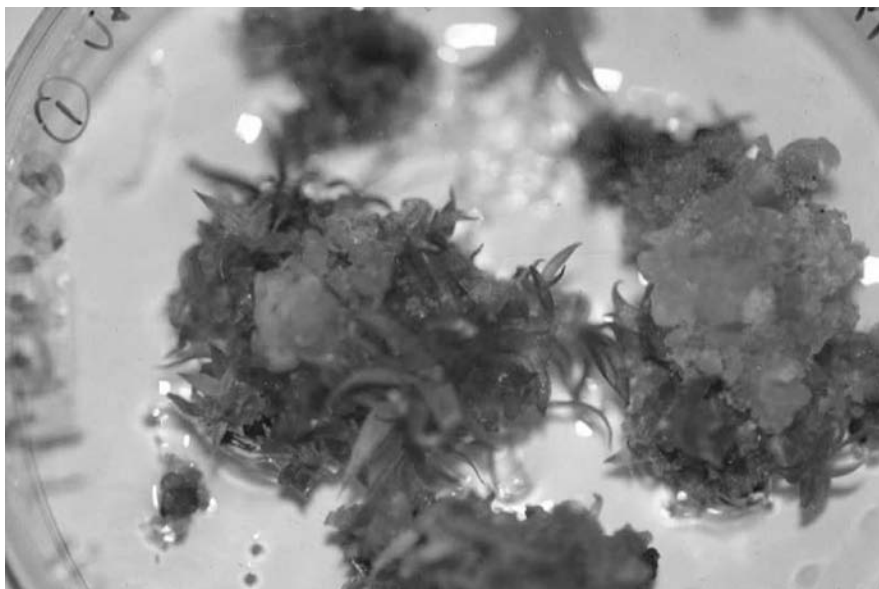


Fig. 5.1.1. Shoot regeneration via organogenesis from callus.

structures. Less commonly, a yellow and friable callus and a soft, mucilaginous, grey-yellow callus were also produced, although neither was morphogenic.

Altman and Ziv (1997) and Teng (1997) regenerated shoots from nodule cultures within 2 weeks of supplementing the medium with 0.5–2.7 μM NAA and 0–0.4 μM BA, with a routine production of plantlets at 6-week intervals.

Regeneration of plants from cell and callus cultures opens up possibilities for the genetic transformation of pineapples; however, these procedures must be viewed very cautiously because of problems with somaclonal variation. According to Scowcroft (1984), as regeneration proceeds from more organized structures (axillary buds) to unorganized tissues (callus), the propensity of cells to undergo genetic changes increases. A preliminary study by Smith *et al.* (2002), however, showed that the percentages of plants showing spininess were similar for plants regenerated from callus cultures compared to plants regenerated from axillary buds. Further studies are needed with larger populations to determine the true extent of somaclonal variation from plants regenerated from cell and callus cultures.

4.1.3. Anther and ovule culture

There is some interest in the regeneration of haploid plants as a first step to circumvent heterozygosity observed in segregating seedling populations. There is doubt whether the development of doubled haploids is likely to be a useful strategy. Other approaches such as those involving some level of inbreeding may be better. To date, limited success has been achieved with ovule culture but anther culture appears to be more difficult (Benega *et al.*, 1996).

4.1.4. Protoplast isolation and culture

Protoplast culture and somatic hybridization may have limited impact on pineapple improvement. Protoplasts have been used in some monocotyledonous species for transformation, but other approaches may be

more appropriate for pineapple. Protoplasts of the cultivar 'Perolera' have been successfully isolated (Guedes *et al.*, 1996), but plant regeneration was not achieved.

4.2. Genetic manipulation

4.2.1. Somaclonal variation

Although pineapple is not generally considered to have an unstable genotype, at least 30 mutants of 'Smooth Cayenne' have been recorded since the early 1920s (Collins and Kerns, 1938). Spiny leaves commonly occur during conventional propagation of 'Smooth Cayenne' and are also observed as a result of micropropagation (Wakasa, 1979, 1989; Smith and Drew, 1990). Spiny variants have also been observed in micropropagated 'Red Spanish' (Liu *et al.*, 1987). A range of other somaclonal variants has been described by Wakasa (1989), and these include variants for leaf colour, leaf shape, waxiness, foliage density and abnormal phyllotaxy. Spininess and dense foliage variants were attributed to chimeras at the donor plant level. Most of the other variation was attributed to regeneration of plants from callus, especially undifferentiated callus derived from the syncarp.

Smith (1988) and Damasco *et al.* (1998) outlined several strategies for minimizing somaclonal variation in micropropagated bananas and all involved regular initiation of cultures while limiting the number of plants multiplied from each explant. While no data exist for pineapple, commercial tissue culture laboratories usually limit multiplication to 300–1000 plants per explant. Roguing of off-types, particularly during nursery establishment, is essential to reduce the percentage of off-types. Many of the somaclonal variants recorded by Wakasa (1989) lend themselves to easy identification and roguing while the plants are quite small. Other less obvious variants, e.g. small, malformed or cracked fruit, require a fruiting cycle for identification. It is particularly important to grow all micropropagated plants through a cycle of fruiting to enable roguing of all somaclonal variants.

Most of the variation reported is either of no benefit or of a deleterious nature for com-

mercial production. We are aware of no reports of useful variants produced from tissue culture. Also we are unaware of mutation breeding programmes for pineapple improvement.

4.2.2. Genetic transformation

Breeding objectives. The pineapple industry worldwide relies upon a single cultivar for processing, i.e. 'Smooth Cayenne'. New cultivars for this market would need to be almost identical to 'Smooth Cayenne' in production, processing and organoleptic qualities. Improvements to this cultivar have not been possible using conventional hybridization techniques, and alternative methods, e.g. genetic transformation, have been advocated in order to make small targeted changes without changing the integrity of 'Smooth Cayenne'. Traits that have been targeted for improvement in 'Smooth Cayenne' include nematode resistance, pineapple mealybug wilt resistance, resistance to fungal diseases (Espinosa *et al.*, 2002), flowering, fruit ripening control and blackheart resistance (Botella *et al.*, 2000; Graham *et al.*, 2000; Rohrbach *et al.*, 2000).

Accomplishments. Transformation of pineapple has been achieved by microprojectile bombardment (biolistics) and by co-cultivation using *Agrobacterium* (Figs 5.1.2 and 5.1.3); however, commercialization and patenting issues have in the past prevented the results of the work from being published. Nan *et al.* (1996) reported the use of the biolistics technique with embryogenic suspension cultures, obtaining low levels of transgenic material. Smith *et al.* (2002) have successfully employed the biolistics technique with callus cultures, achieving an average of 1% transformation efficiency. On the other hand, Firoozabady and Gutterson (1998) produced transgenic pineapples using the *Agrobacterium*-mediated technique with embryogenic cultures, and Isidron *et al.* (1998) have utilized a similar method. Graham *et al.* (2000) have successfully used *Agrobacterium* to transform 'Smooth Cayenne' leaf bases, resulting in the production of organogenic cultures and transgenic plants, with transformation efficiency levels of approximately 2%. Espinosa *et al.* (2002) transformed morphogenic callus of 'Smooth Cayenne' with *Agrobacterium* strains AT2260 and LBA4404 containing gene constructs



Fig. 5.1.2. A field planting of transgenic pineapples.

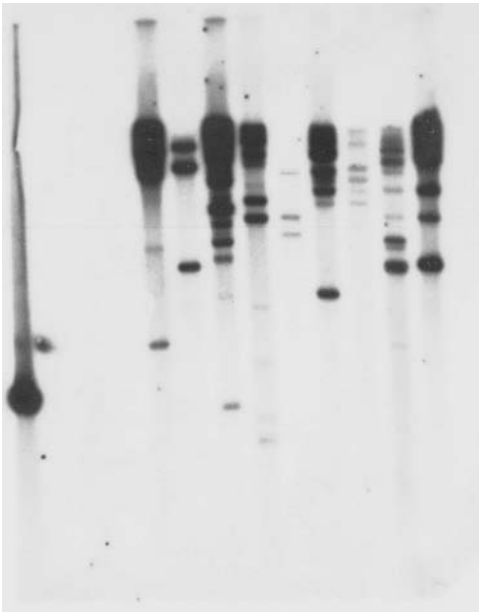


Fig. 5.1.3. Southern blot analysis of *nptII* transformed pineapple lines. Lane 1 – *nptII* probe; lane 2 – empty lane; lane 3 – molecular weight marker; lane 4 – control DNA (micropropagated plants); lanes 5–13 – lines from independent transgenic events via biolistic transformation.

pHCA58 and pHCG59, respectively, and observed transformation efficiency of 6.6%. The plasmid pHCA58 contained a class I bean chitinase gene under the control of a hybrid *ocs*-cauliflower mosaic virus (CaMV) 35S–rice actin I promoter (pA5) and the tobacco ap24 gene under the control of the CaMV 35S promoter. The plasmid pHCG59 contained the chitinase gene under the control of the hybrid pA5 promoter and a class I tobacco β -1,3-glucanase gene under the CaMV 35S promoter.

Selection of transgenic material is achieved by co-introducing the gene of interest with a marker gene, e.g. conferring resistance to an antibiotic (Dekeyser *et al.*, 1989); Caplan *et al.*, 1992) or herbicide (Vasil *et al.*, 1992; Cole, 1994; Dennehey *et al.*, 1994). Graham *et al.* (2000) observed that 50 μ g/ml geneticin and 20 μ g/ml hygromycin are useful for selection, but not kanamycin, even at concentrations as high as 500 μ g/ml. Promoter elements and constructs developed

for use in other species have been adapted for use with pineapple. The maize ubiquitin and the CaMV 35S promoters function well in transient assays in pineapple fruit, leaves and callus (Graham *et al.*, 2000). The transformation technique developed by DNA Plant Technology Inc. (DNAP) in the USA utilizes these two promoters (Firoozabady and Gutterson, 1998). However, confirmation of the genuine utility of these promoters in transgenic pineapple will await the generation and analysis of stable, transformed lines. Similarly, for driving expression of the *nptII* selectable marker gene, both the *Ubi-1* and CaMV 35S promoters and promoters derived from segments of the subclover stunt circovirus have been successfully used to create stable transformants (Graham *et al.*, 2000).

4.3. Germplasm conservation

Advances in breeding and genetic engineering require that the genetic diversity of pineapples and their close relatives should be preserved and used for pineapple improvement. While tissue culture techniques offer the opportunity for rapid propagation, they also offer the convenience of medium- and long-term storage of germplasm and facilitate its safe distribution.

Lower temperatures (16–20°C) have been used to extend subculture times for up to 4 years (Sugimoto *et al.*, 1991). Zee and Munekata (1992) observed that reducing the nutrient salts of MS medium to one-quarter was successful for medium-term (12 months), low-input maintenance of pineapple cultures. For long-term storage, cryopreservation has been utilized. Gonzalez-Arnao *et al.* (1998) described optimal conditions, which included the following: (i) 2-day pre-culture of shoot apices on semi-solid MS medium with 0.3 M sucrose; (ii) 25 min exposure to cryoprotectant solution containing glycerol (1 M) and sucrose (0.75 M); and (iii) dehydration with plant vitrification solution 2 (PVS2) (30% (w/v) glycerol + 15% (w/v) dimethyl sulphoxide (DMSO) + 0.4 M sucrose) at 0°C for 7 h before immersion in liquid nitrogen.

5. Conclusions

Micropropagation has been adopted for commercial production of pineapple, although reliable methods are required for the control and detection of deleterious off-types that are produced *in vitro*, and before plants are grown in the field. It is probable that genetic engineering will become an established method for pineapple improvement, particularly for cultivars such as 'Smooth Cayenne' that do not lend themselves readily to improvement by conventional breeding

strategies. Molecular markers have become powerful tools in pineapple taxonomy and will assist with more precise identification and classification. They will also become extremely useful in hybridization programmes for the selection of parental combinations containing important traits, thus reducing the number of generations required, or for screening populations for specific traits, thereby reducing evaluation time. Molecular markers also have potential for gene identification for subsequent isolation, replication and sequencing.

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6

Caricaceae

The *Caricaceae* is a small family consisting of 22 species in six genera. All of the genera are from South and Central America except for *Cylicomorpha* Urban from West Africa. *Jarilla* Rusby contains three species, *Jacaratia* A. DC. seven species and *Horovitzia* Badillo a single species. The American genera *Carica* and *Vasconcellea* are found from southern Mexico to the Andean highlands. While *Carica papaya* is dioecious or gynodioecious, *Vasconcellea* spp. are dioecious

except for the monoecious *V. monoica* and the dioecious and monoecious *V. cundinamarcensis* (Horovitz and Jimenez, 1967; Badillo, 1971). *C. papaya* is the most important crop taxon of the family; however, *V. cundinamarcensis* and a natural parthenocarpic hybrid, *V. heilbornii-badillo* var. *pentagona* ('babaco'), are also consumed fresh, roasted, as juice and as marmalade and preserves (Van den Eynden *et al.*, 1999).

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6.1 *Carica papaya* Papaya

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1. Introduction

1.1. Botany and history

The papaya most probably originated along the Caribbean coast of Central America (Manshardt, 1992). According to isozyme analysis, the greatest diversity in the species occurs in the Yucatan–San Ignacio–Peter–Rio Motagua area of Central America, with the wild populations having greater diversity than the domesticated ones in the same area (Morshidi, 1996). Badillo (2000, 2001) placed papaya in a monotypic genus of the dicotyledonous family *Caricaceae*, transferring its 22 closest relatives (Badillo, 1971, 2000) to an older taxon, *Vasconcellea* St Hil., citing differences in chloroplast DNA data (Aradhya *et al.*, 1999), the enclosed locular seed structure of the fruit in the other species compared to *Carica papaya* and breeding incompatibilities (Sawant, 1958; Horovitz and Jimenez, 1967; Mekako and Nakasone, 1975; Manshardt and Wenslaff, 1989a,b).

The shape of papaya plants is reminiscent of palmate-leaved, large monoaxial palms. Young plants are single stemmed during the first 1–2 years of growth, although in highly fertile orchards heavy lateral branching develops early on juvenile plants. Mature papaya leaves are palmate with deep lobes, supported by smooth, hol-

low petioles that leave highly textured leaf scars on an otherwise smooth, light tan, hollow stem. The persistent leaf scars enlarge as the plant grows in circumference. The generation time of this large herbaceous species is short, from 8 to 12 months from seed to ripe fruit. Lateral branches develop after the first fruit begin to mature, but the tree can return to monoaxial growth after high winds cause heavy, fruit-laden lateral branches to fall off. The trees differ in sex type; pistillate and hermaphrodite plants bear fruit year-round in warmer tropical regions while staminate plants usually produce no fruit. The flowers of pistillate and hermaphrodite plants are borne in short cymes but staminate trees develop highly elongate cymose panicles (see Storey, 1953).

Papayas (*C. papaya* L.) are consumed year-round in the tropics and subtropics. The numerous seeds, when dried, remain viable for several years, and this facilitated their movement from the Caribbean region to Malacca and to India (Storey, 1941) in the 1600s (Purseglove, 1968). From Malacca and/or the Philippines they were dispersed throughout Asia and the South Pacific. Don Francisco Marin, a Spanish explorer and horticulturist, carried the papaya to Hawaii from the Marquesas during the early 1800s. They became an export crop of Hawaii in 1948 (Yee *et al.*, 1970).

1.2. Importance

Ripe papaya fruit resemble melons and are rich in vitamins A and C (Arriola *et al.*, 1980; Wenkam, 1990) and calcium (Wenkam, 1990). They are consumed at breakfast or as dessert, or the immature fruit is prepared in fresh salads or cooked in stews in Asia. The latex contains the protease papain, the major component of meat tenderizer, and is collected from scored green fruit in some African countries, e.g. Tanzania, and in India (Nakasone and Paull, 1998). Papain is used as a digestive medicine in the pharmaceutical industry, for clearing beer, in tanning, in the cosmetics industry and in the manufacture of chewing gum (Nakasone and Paull, 1998).

Papayas are ranked in world production tonnage at about the same level as dates, but higher than strawberries and grapefruit (FAOSTAT, 2004). Approximately 6.35 Mt of papayas are produced annually (FAOSTAT, 2004). Brazil has been the largest producer, with 1.5 Mt in 2002, followed by Nigeria, India and Mexico. Hawaiian and Philippine papayas are sold in Japanese markets, and Hawaii, Mexico, Belize and Brazil compete for US mainland markets; Brazil supplies the European Union (EU) market. Most papaya-growing regions are in the warm tropics and subtropics and the hermaphrodite type is primarily grown.

1.3. Breeding and genetics

Papayas are polygamous diploids with nine pairs of chromosomes (Storey, 1953). One pair of chromosomes separates precociously at anaphase (Kumar *et al.*, 1944) and is believed to be associated with sex. Storey (1953, 1958, 1976) described several papaya sex types, which make breeding very complex. There are two basic alleles for sex type, the recessive *m* and the dominant *M*, which is represented by M_1 and M_2 , two alternative forms of the male allele in pollen-bearing or staminate plants. Female or pistillate plants are designated *mm* while staminate plants are *Mm*. There is no true male type, *MM*. The homozygous male is apparently lethal since approximately 25% of

mature seed coats are empty (Storey, 1953). The two staminate types of papayas are dioecious (M_1m) and gynodioecious (M_2m).

Sex expression in progeny of the dioecious papayas, M_1m , consists of pistillate and staminate trees. Pollen-bearing plants produce large, fragrant panicles of slender, tubular staminate flowers with vestigial ovaries and are often referred to as 'males'. These trees can at times produce fruit in a panicle from one or more perfect flowers having functional stamens and an ovary. The fertile flowers are called teratological staminate types. Female flowers of both dioecious and gynodioecious papayas are plump with non-tubular corollas of separated petals.

The second type of papaya is the gynodioecious M_2m type. When self- or cross-pollinated the M_2m hermaphrodites produce females and hermaphrodites. Hermaphrodites have self-pollinating perfect flowers, called elongata, which are 95% self-fertilized while the petals are still unfurled. Self-pollination results in inbreeding for stabilizing genetic traits to homozygosity. Elongata flowers are tubular like the staminate types but thicker in diameter to accommodate the functional ovary. Hermaphrodite trees, in the warmer seasons, develop functional male flowers of three types: staminate, teratological staminate and reduced elongata, in which the ovary is underdeveloped in addition to or in place of elongata flowers. Fruit set is low or nil if the elongata type is not produced. Three types of functional female flowers develop on hermaphrodite trees, usually in response to cooler weather:

1. Carpelloid elongata, in which one to five of the stamens in the inner whorl become transformed into carpels.
2. Pentandria, a perfect flower but with only one whorl of stamens and loss of the original carpels. The fruit that develop from pentandria have five to ten locules and resemble females. Pentandria flowers often develop on vigorous young and well-fertilized older trees (Awada and Ikeda, 1957; Awada, 1958).
3. Carpelloid pentandria, in which the inner carpel aborts and one to five stamens of the outer whorl become carpeloid and fuse with the inner carpeloid stamens.

The staminate genes or factors apparently have a strong influence over the female and environment/nutrition cues affect flower form. If all five outer whorl stamens become carpeloid, the bud and fruit resemble a female; however, when sliced in cross-section ten carpels, sometimes separated by cross walls, can be seen. The shape of dioecious and gynodioecious female fruits is unaffected by age, season and nutritional status. Carpellody is never a problem because stamens never form in female flowers. Therefore, the receptors for environmental/nutritional cues lie in the 'male' sections of the genome, which are not present in the pistillate plants.

Self-pollinated hermaphrodites or crosses within, among and between the dioecious and gynodioecious papayas result in differing ratios of sex types in offspring (Storey, 1953). Females from either dioecious or gynodioecious papayas when pollinated with either type yield staminate plants of the pollen parent type in a 1 female : 1 pollen parent ratio. F_1 hybrids are usually made by this type of cross rather than by emasculation of the staminate flowers because the risk of self-pollen contamination is high. Dioecious staminate trees with the occasional teratological perfect flower will produce seedlings with sex ratios of 2 staminate:1 female. The Australian

papaya-breeding programme depends on self-pollination of the occasional teratological staminate flower to achieve homozygosity of desired traits (Manshardt, 1992).

The wild papayas in southern Mexico bear small dioecious fruit with thin flesh and numerous seeds (Manshardt, 1992; Morshidi, 1996). Selection by humans has resulted in the thick-fleshed fruit. Dioecious and hermaphrodite types are both found in the centres of diversity, but Manshardt suggested that hermaphrodites, being self-pollinated, were probably selected because fruit production was more reliable.

1.3.1. Major breeding objectives

Disease resistance, increased yields and improved quality and storage traits are the most important breeding objectives (Nakasone and Paull, 1998). Consumer preferences for flesh colour, flavour and fragrance, shape, firmness and size are perennial standards that invariably change after new cultivars are introduced.

Papaya ringspot virus (PRSV) resistance.

Papaya ringspot virus (PRSV; Fig. 6.1.1) is the most important limitation of papaya production worldwide (Gonsalves, 1998) and can destroy a crop if isolation and roguing



Fig. 6.1.1. Papaya ringspot virus (PRSV) symptoms (severe) on a susceptible plant.

are not implemented. PRSV is a member of the potyvirus family of single-stranded RNA viruses transmitted by several aphid vectors (Purcifull *et al.*, 1984). Strains of the virus are found in nearly every papaya-growing region (Gonsalves, 1998).

PRSV was first reported in Hawaii in 1945 (Jensen, 1949), and became a serious threat to the papaya industry in the mid-1950s when the industry was based on Oahu (Yee *et al.*, 1970). With no genetic resistance, the industry relocated in the late 1950s to the island of Hawaii, and flourished there for 30 years (Gonsalves, 1998). The Hawaiian production nearly collapsed in 1997 following an outbreak of PRSV in the major growing area 5 years earlier (Gonsalves, 1998). Cross-protection with a mild strain of PRSV (Yeh and Gonsalves, 1984; Wang *et al.*, 1987) provided limited protection for certain cultivars (Mau *et al.*, 1989) but was not used to stem the 1992 outbreak. Other viruses, including papaya mosaic virus and papaya apical necrosis, have not caused major epidemics (Gonsalves, 1998).

Fungal and oomycete disease resistance.

Fungal/oomycete diseases, a group that includes *Phytophthora* fruit, stem and root rots (*Phytophthora palmivora*), anthracnose (*Colletotrichum gloeosporioides*), powdery mildew (*Oidium caricae*) and black spot (*Asperisporium caricae*) (Nishijima, 2002), cause serious production and postharvest losses. Fruit rots that are caused by *Rhizopus* sp., *Stemphyllium* sp., *Phomopsis* sp., etc. are important postharvest diseases, and are controlled by preharvest fungicide treatment and by postharvest disinfestation with forced hot air (Nishijima, 1994; Armstrong *et al.*, 1995).

Other diseases. Phytoplasma-associated diseases, including papaya dieback, yellow crinkle and mosaic (the latter two may be symptoms of the same disease complex), are production problems in Australia. Tolerant lines have not been reported in the literature (Guthrie *et al.*, 1998). Control measures have included ratooning of dieback-affected plants (the disease is localized in the apex) and removal of crinkle- and mosaic-symptomatic plants.

Resistance to insects and other pests.

Arthropod pests pose threats pre- and postharvest. *Myzus persicae* (Namba and Kawanishi, 1966) was reported to be the major vector in Hawaii but its control did not prevent spread of virus infection. Other aphid species are known vectors, including *Aphis gossypii* and *Aphis citri* (Purcifull *et al.*, 1984). The oriental fruit fly *Bactrocera dorsalis* (Hendel), Mediterranean fruit fly, *Ceratitidis capitata* (Wiedemann), and melon fly, *Bactrocera cucurbitae* (Coquillett), oviposit in ripening papaya fruits and pose a threat to the California fruit industry. All Hawaii papayas entering California are either forced-vapour heat-treated or irradiated at levels that are lethal to these pests (Armstrong *et al.*, 1995; Follett, 2000). The white peach scale, *Pseudaulacaspis pentagona* (Targioni-Tozzetti), was discovered in Hawaii in 1997. The pest is controlled with Sunoil with limited success (Follett, 2000). The carmine mite, *Tetranychus cinnabarinus*, is the most important acarid pest of papayas, followed by the papaya leaf edgeroller mite, *Calacarus brionese*. Red and black mite, *Brevipalpus phoenicis*, is an occasional pest. Effective miticides include sulphur, avermectin and Vendex but control is costly. Leafhoppers, *Empoasca stevensi*, are serious pests, and phytotoxicity causes margins of leaves to become chlorotic. Insecticides imidachloprid, pyrethrin and malathion are used to control infestations (Follett, 2000).

Fruit and plant characteristics. A major breeding objective for the subtropics of Queensland, Australia, is a reddish-orange fleshed, musk-flavoured fruit (Chay-Prove, 1997). Shorter bearing height is also desired to allow earlier harvesting and harvesting for a longer period without additional harvesting devices that incur higher costs. Cold tolerance and higher soluble solids during winter are important in South Africa (Louw, 1999).

Introgression of cold tolerance from wild species. The *Vasconcellea* spp. of the Andean region include genotypes that could be used as new crops and/or hybrids in

cooler regions (Scheldeman *et al.*, 2002). Although breeding studies with papaya have revealed post-fertilization problems (Manshardt and Wenslaff, 1989b), crosses between some of these dioecious species have been successful (Jimenez and Horovitz, 1958; Mekako and Nakasone, 1975). 'Babaco', a sterile hybrid, is propagated as cuttings, has been grown in Australia and other temperate locations and is sold in markets in that country as well as in Europe.

1.3.2. Breeding accomplishments

PRSV resistance. Genetic PRSV resistance does not exist in papaya cultivars (Mekako and Nakasone, 1975). An accession from Colombia was used by Zee (1985), Conover *et al.* (1986) and V. Prasartsee (Gonsalves, 1998) to introgress tolerance into cultivars in Hawaii, Florida and Thailand, respectively. The dioecious 'Cariflora' and recurrent selections from hybrids with 'Khakdum' and 'Khaknuol' were introduced into commerce in Florida and Thailand, respectively. 'Sinta' was developed from 'Cavite', a Philippine PRSV-tolerant line (P. Magdalita and V. Villegas, personal communication). Taiwan developed F₁ hybrids, 'Tainung #2' and 'Tainung #5', from large Thai papayas and 'Sunrise', (S.-D. Yeh, personal communication). 'Tainung #5' and another Taiwanese cultivar, 'Red Lady', have some PRSV-tolerance. In the US Virgin Islands, Caribbean and Indian PRSV-tolerant papaya lines have been identified, e.g. dioecious 'Washington' from India, and tolerant hybrids with fair horticultural traits have been released (T. Zimmerman, personal communication).

PRSV-resistant wild species have been reported (Malaguti *et al.*, 1957; Riccelli, 1963; Conover, 1964; Horovitz and Jimenez, 1967; Adsuar, 1971). Several researchers have attempted to introgress resistance genes into papaya and other susceptible species (Sawant, 1958; Horovitz and Jimenez, 1967; Mekako and Nakasone, 1975). Mekako and Nakasone (1975) produced six interspecific *Vasconcellea* hybrids, but none with papaya. Khuspe *et al.* (1980) recovered three seedlings of *C. papaya* × *V. cauliflora*, but lost them

before confirming their hybrid nature; embryo culture was utilized to rescue the hybrid embryos that developed in the absence of endosperm. Litz and Conover (1981b, 1982) cultured ovules from the same cross and confirmed by isozyme analysis that the plants were hybrids (Moore and Litz, 1984). F₁ hybrids between papaya and *V. cundinamarcensis*, *V. quercifolia*, *V. stipulata* and *V. cauliflora* were recovered (Manshardt and Wenslaff, 1989a,b). Field resistance was demonstrated in the *V. quercifolia* and *V. cauliflora* crosses, but the plants were nearly sterile. Chen *et al.* (1991) described secondary somatic embryos from rescued embryos of papaya × *V. cauliflora* hybrids that were verified by isozyme analysis. Magdalita *et al.* (1996, 1997a, 1998) used highly viable pollen from *V. cauliflora*, which was produced during the spring, summer and autumn, and approx. 94% of the hybrid embryos germinated and normal-looking plants were recovered. *V. cauliflora* and its hybrids with papaya were shown to be resistant to Australian PRSV isolates (Magdalita *et al.*, 1997b); however, fertility of the hybrids was low (Drew *et al.*, 1998). Fertile interspecific hybrids between papaya and *V. quercifolia* have been produced (Drew and O'Brien, 2001). The improvement in fertility was attributed to the relative genetic closeness of papaya and *V. quercifolia*, as determined by isozyme and randomly amplified polymorphic DNA (RAPD) analysis (Jobin-Décor *et al.*, 1997a,b). Interspecific F₁ hybrids were back-crossed with papaya to create fertile PRSV-resistant plants. A single fertile back-cross clone survived six inoculations and 9 months under virus pressure in the field before it became infected (R. Drew, unpublished results).

Fungal/oomycete tolerance. 'Waimanalo' was developed for tolerance of *Phytophthora* root and fruit rot (Nakasone and Aragaki, 1975). 'Kamiya', a selection from 'Waimanalo', and two other oomycete-tolerant cultivars, 'Saipan Red' and 'Line 40', were hybridized and crossed with 'Rainbow' to introgress PRSV resistance into the hybrids and to further combine quality and resistance traits (R. Manshardt, personal communication). Selections of F₃ progeny showed

Phytophthora resistance and quality traits. While breeding for tolerance resulted in somewhat improved cultivars, control of these diseases still requires fungicide application (Nishijima, 1994). Application of 15 mM salicylic acid (SA) and 1.0 mM benzo (1,2,3)-thiodiazole-7-carbothioic acid S-methyl ester (BTH) can enhance systemic acquired resistance (SAR) in papaya (Zhu *et al.*, 2000a). The activities of the pathogenesis-related (PR) proteins, chitinase and β -1,3-glucanase, increase more than six-fold following BTH treatment, although the treatment is slightly inhibitory to papaya seedlings.

Other traits. Excellent cultivars have been developed in Asia by conventional breeding (Chan, 2002). Fruit yield is significantly impacted by the interaction of genotype with the environment (Chan and Mak, 1996). In Malaysia, 'Sunrise' was hybridized with 'Subang 6' (non-recurrent parent), a selection from a farmer's field (Chan, 2002), to produce 'Ekstotika', a papaya with improved fruit size and adaptation (Chan, 1987). 'Ekstotika II' is an improved F_1 hybrid between line 19 and line 20 (Chan, 2002).

In Thailand, the combining ability of three papaya cultivars has been reported (Subhadrabandhu and Nontaswatsri, 1997). Various characters were measured, including days to flower, height of flowering, circumference at first flowering, position of first fruit, number of nodes to the first fruit, number of fruits for each crop for each plant, fruit weight, fruit length and width, per cent of fruit cavity, flesh thickness and firmness, % total soluble solids (TSS), number of seeds in each fruit, etc. Significant specific combining ability was found for all characters except the number of fruit for each crop for each plant and number of seeds for each fruit. Significant differences between reciprocal crosses were found which indicated a maternal effect on fruit weight. Quality of F_1 to F_3 hybrid papayas was compared with the parents for improved eating quality for local and export markets (Somsri and Khaegkad, 2002). Twenty-two F_1 hybrids with Australian ('Richter' and '2.001'), Thai ('Khakdum') and Hawaii ('Sunset') cultivars produced 170 F_3 , F_2 , F_1 and parent hermaphrodite plants, which were compared. Eating

quality ranged from better than the parents to intermediate. Most TSS percentages were intermediate or higher than the sweeter parent. The F_3 s had the most improved eating quality, especially when 'Sunset' was one of the parents. The % TSS was negatively correlated with fruit weight and flesh thickness; the smaller the fruit, the thinner the flesh and the greater the sweetness. Forty selections were recommended for micropropagation and recurrent selection in Thailand.

'Petersen', 'Sunnybank' and 'Arlene/57' are yellow Australian dioecious cultivars with higher sugar content, blemish-free skin and tolerance of cold (Nakasone and Paull, 1998). 'Sinta' ('Sweetheart'), a large-fruited type, has been developed in the Philippines for the local market (P. Magdalita, personal communication). 'Waimanalo' is tolerant of *Phytophthora* and has a low bearing height (Nakasone and Aragaki, 1975). 'Sunrise' and the sibling line 'Sunset' are low- and early-bearing cultivars. 'Hortus Gold' and 'Honey Gold' were developed in South Africa (Nakasone and Paull, 1998).

2. Molecular Genetics

Molecular techniques have been used to isolate and study genes that are important for papaya disease resistance and plant growth and development. Genomics, using linkage, fine and physical mapping, DNA fingerprinting, marker-assisted selection and functional genomics, could be used to manage papaya breeding programmes aimed at understanding environmental adaptation, efficiency of physiological processes, disease resistance, growth and yield parameters and sex expression (Manshardt, 1992).

2.1. Gene cloning

2.1.1. Control of fruit ripening

Genes that are involved in papaya fruit ripening have been cloned (Neupane, 1997; Neupane *et al.*, 1997; Zhou *et al.*, 1998; Lee *et al.*, 1999, 2001; Chen *et al.*, 2001; Laurena *et al.*, 2002; Chen and Paull, 2003).

Aminocyclopropane carboxylase (ACC) synthase and oxidase have been cloned by reverse transcriptase polymerase chain reaction (RT-PCR) using six degenerate oligonucleotides from highly conserved ACC synthase and oxidase domains (Neupane, 1997; Neupane *et al.*, 1997). The ACC synthase gene was determined to be a single copy, whereas the ACC oxidase gene is a member of a multigene family. The nearly full-length clones were sequenced and the ACC synthase gene has been transformed into papaya in the sense and antisense orientations. Leaves of two plants expressed the antisense message in Northern blots; however, due to the small number of transgenic plants recovered, delayed ripening was not observed. Laurena *et al.* (2002) cloned two ACC synthase genes from ripe fruit by RT-PCR.

A single copy ACC oxidase gene, CP-ACO2, has been cloned and sequenced (Chen *et al.*, 2001). It has 77% homology with CP-ACO1 and is associated with late-stage fruit ripening and leaf senescence. CP-ACO2 is induced during fruit ripening and leaf senescence while CP-ACO1 is induced before colour break, the early ripening stage of mature green fruit. CP-APO2 is an ethylene- and wound-inducible gene. Two distinct pathways for ACC oxidase regulation of papaya ripening have been proposed: during maturation and during late-stage ripening.

Papaya ripening-related genes have been cloned by differential display (Lee *et al.*, 1999, 2001). A cell wall invertase gene from papaya fruit has been cloned and its expression level has been determined (Zhou *et al.*, 1998). Cloning and expression of xylanase have been reported (Chen and Paull, 2003). The xylanase gene could be utilized to control growth, abscission, dehiscence and/or fruit ripening characteristics.

2.1.2. Fungal/oomycete tolerance

Genes for fungal/oomycete tolerance have been cloned using PCR with degenerate primers for the pathogenesis-related protein 1 (PR-1) conserved peptide sequence GHYTQVW. Four partial complementary DNA (cDNA) clones of PR-1-like genes, A,

B, C and D, have been cloned (Qiu *et al.*, 2002, 2003a; Qiu, 2003). PR-1B and D genes have been induced by BTH, a salicylic acid analogue that induces SAR in papaya (Qiu *et al.*, 2003b), but they were not induced by *P. palmivora* root drench inoculation (Qiu *et al.*, 2003c). The oomycete caused peroxidase mRNA levels to increase significantly; however, expression of PR-1A, chitinases and osmotin-like protein was not enhanced (Qiu *et al.*, 2003c). Papaya NPR1, the PR protein controller gene (Cao *et al.*, 1998), has also been cloned by PCR, using consensus sequences from *Arabidopsis*, tobacco and tomato (Qiu, 2003). The NPR1 gene has an ankyrin repeat region and a possible nuclear localization sequence, both of which are essential for NPR1 function in *Arabidopsis*. The amino acid sequence of the papaya NPR1 homologue shares 71% with rice and 66.8% with *Arabidopsis*. A SAR cDNA subtraction library has been constructed and genes up-regulated after BTH treatment of seedlings have been cloned (Qiu, 2003; Qiu *et al.*, 2003b,c). Differential screening has been used to locate 26 randomly selected clones, seven of which have been confirmed by Northern blot analysis to be up-regulated by BTH. The clones include cDNAs for PR-1 protein, two chitinases, one osmotin-like protein, peroxidase and cytochrome 450. The expressed sequence tags (ESTs) for the up-regulated genes are being used to study the defence responses of papaya to various pathogens. The papaya NPR1 gene has been introduced into papaya to determine if transgenic plants would exhibit improved disease resistance (Qiu *et al.*, 2003a,b).

2.1.3. Flower development

Some genes associated with flower development have been cloned and characterized (Yu *et al.*, 2003a,b). Genes homologous with those involved with *Antirrhinum* and *Arabidopsis* carpel development have been cloned. The *Arabidopsis* C class flowering gene *AGAMOUS* has a single papaya homologue, *PAG*, which shares 85% homology within MADS (MCMI, *AGAMOUS*, *DEFICIENS*, *SRF* (serum response factor)) box

genes and K box domains. The gene is expressed at high levels in carpels. The *Arabidopsis* gene *LEAFY*, a positive regulator of *AGAMOUS*, also has a single papaya homologue, *PFL*, with which it shares 65% homology. The *PFL* protein shares 71% homology with *Platanus racemosa* and *Populus trichocarpa*. *PFL* exists in a single copy in the papaya genome and is expressed at low levels in the apical meristems of young seedlings but at high levels in mature floral meristems. The second intron of *PAG* is being subcloned and sequenced. *Hua1*, a regulator of stamen and carpel development, has been cloned (Yu *et al.*, 2003b), and shares 82% homology with *Arabidopsis*.

2.1.4. Other genes

The 5S ribosomal RNA gene repeat units from papaya and *V. quercifolia* have been cloned, using PCR, and partially characterized (Singh and Yadav, 2002). The organization of the 5S genes was studied. Six amplified products, ranging from 300 to 2000 bp, have been obtained and sequencing of the clones is planned.

A small guanosine triphosphate (GTP)-binding protein, *pgp1*, was cloned from papaya cDNAs during the establishment of papaya ESTs (Urasaki *et al.*, 2000), and an EST clone, *pRA4-3*, encoding the partial sequence of *pgp1* was obtained. Southern analysis showed that there were several *pgp1*-related genes in papaya. The gene was expressed equally in seedling stems grown in the light and dark, and was therefore not involved in phytochrome-mediated signal transduction as an auxin signal transducer in seedling stems.

A cysteine proteinase gene, *CC23*, was purified from *V. cundinamarcensis* latex and a partial putative genomic fragment was cloned that showed strong homology with chymopapain isoform IV from papaya (Pereira *et al.*, 2001). The translated sequence was similar to chymopapain isoform II (73%) and *CC-III* (77%) from *V. cundinamarcensis*. The papaya prepapain gene was expressed in a yeast expression/secretion system (Ramjee *et al.*, 1996). Yields were tenfold higher than in other yeast or bacterial expression systems.

Cystatins are cysteine proteinase inhibitors that are implicated in nematode resistance in plants (Urwin *et al.*, 1998). A papaya cystatin gene has been isolated from a cDNA library of papaya leaves and characterized (Song *et al.*, 1995). The protein is 11,282 Da with > 40% identity with other published plant cystatins. The enzyme contains a single Cys residue and has a variation in the papain-binding region. Over-expression of the papaya cystatin could improve nematode tolerance in the crop.

The optimum temperature range for papaya growing is 21–33°C (Nakasone and Paull, 1998). *Vasconcellea* spp. grow at 3000 m above sea level and can tolerate frost. The cold tolerance gene C-repeat/dehydration-responsive element binding factor (CBF) from *Arabidopsis* (Jaglo-Kirsten *et al.*, 2001) was used to clone a homologue from the mountain papaya, *V. cundinamarcensis* (S. Dhekney and R. Litz, unpublished results). The gene was sequenced and will be transformed into papaya to attempt to improve its cold tolerance in subtropical and warm temperate regions.

2.2. Genomics

According to Hofmeyr (1938), there is a weak linkage between purple stem and sex and recessive yellow flower colour and sex. Storey (1953) listed several papaya mutants, but was unable to find linkages with sex expression. Papaya, with its relatively small genome (372 Mb/1C) (Arumuganathan and Earle, 1991) and short generation time (c. 1 year), is well suited to be a model for genomics research. Breeding could be enhanced with molecular markers that are correlated with useful traits. Genomics research has focused on development of: (i) a repository of sections representing the chromosomal complement of the entire genome; (ii) a linkage map of as many markers as possible using segregation of polymorphisms between parental traits; and (iii) a physical map consisting of DNA sequences of the markers (R. Ming, personal communication).

The long-term objectives of genomics research are to study genome structure,

organization and content of papaya, using a large insert bacterial artificial chromosome (BAC) library. A BAC library has been established from transgenic 'SunUp' (Ming *et al.*, 2001), and consists of 39,168 clones with an average insert size of 132 kb or $13.7 \times$ the papaya genome equivalent. Approximately 92% of the papaya genome is believed to be in this library. It has been used for cloning meristem and floral organ identity genes and to clone the sex determination gene (Ming *et al.*, 2003).

2.2.1. Linkage maps

Sondur (1994; Sondur *et al.*, 1996) established the first RAPD linkage map of papaya, and reported a marker for sex expression. The map was created by amplifying genomic DNAs from a segregating population of 'Line 356' $\times$ 'Sunrise', using anonymous primers, and segregating F_2 trees. A linkage map has been constructed using amplified fragment length polymorphisms (AFLPs) (Ma, 2003; Ma *et al.*, 2003; Ming *et al.*, 2003). The locations of the PRSV resistance transgene, sex type morphology and fruit flesh colour have been mapped with log of odds (LOD) scores of 5.0 and recombination frequencies of 0.25. Each linkage group contained 27 to 414 markers, with lengths of 86.2–865.2 cM. The total length of the map is 3464.1 cM with an average distance between markers of 2.3 cM.

According to Storey (1938, 1953), recombination is highly suppressed around the sex locus. The cytosines are highly methylated. A larger number of sex co-segregating markers are generated from AT-rich rather than GC-rich primers. By far the largest marker cluster surrounds the sex locus. The sex co-segregating marker W11 (Deputy *et al.*, 2002) hybridizes with four BAC clones and has stimulated studies to clone the sex determination gene (Ma, 2003).

2.2.2. Markers

Early linkage maps were generated based on phenotype for three genes (Hofmeyr, 1938) and RAPDs (Stiles *et al.*, 1993; Sondur, 1994; Sondur *et al.*, 1996). The RAPD map con-

sisted of 62 markers on 11 linkage groups. The markers had LOD scores of at least 4.0. One marker segregated for sex expression in F_2 plants from a 'Line 356' (derived from 'Cariflora') $\times$ 'Sunrise' hybrid. The marker has been used to construct sequence-characterized amplified region (SCAR) primers (Deputy *et al.*, 2002), which, in conjunction with a marker common to both females and hermaphrodites (T1), are diagnostic for identification of hermaphrodite seedlings. Other PCR-based sex markers have been reported (Somsri *et al.*, 1998; Parasnis *et al.*, 2000; Lemos *et al.*, 2002; Urasaki *et al.*, 2002). Recombination frequency was zero. Liu *et al.* (2004) provided evidence from non-recombining GC-rich regions with low gene content to support Storey (1938, 1953) and Hofmeyr (1967) that the lack of recombination is due to the presence of an ancient male Y sex chromosome. Chromosome walking and fine mapping have been used to locate the sex locus (Liu *et al.*, 2004).

Phenotypic data having probable quantitative trait loci (QTLs) have been gathered and DNAs have been collected from 95 F_2 individuals having traits in the extreme ranges of fruiting height (R. Ming, personal communication). The AFLPs are being used to fingerprint the population and correlations with the QTLs will be made. AFLPs have been used to fingerprint papaya and other species (Kim *et al.*, 2002; Van Droogenbroeck *et al.*, 2002). Relationships from breeding records have been corroborated, although some cultivars have varied depending on the source of the materials.

Using molecular markers, the genetic similarity between papaya and six *Vasconcellea* spp. has been shown to be low, while the genetic relationships among the *Vasconcellea* spp. have been shown to be close, in agreement with Jobin-Décor *et al.* (1997a,b), Aradhya *et al.* (1999) and Van Droogenbroeck *et al.* (2002). The most closely related species are *V. cundinamarcensis* and *V. stipulata* (Sharon *et al.*, 1992; Jobin-Décor *et al.*, 1997a,b; Kim *et al.*, 2002; Van Droogenbroeck *et al.*, 2002). The relatedness of papaya with *Vasconcellea* spp. has been studied using AFLP (Vos *et al.*, 1995) on 95 accessions (Van Droogenbroeck *et al.*,

2002). This study confirmed earlier RAPD and isozyme (Jobin-Décor *et al.*, 1997a,b) and chloroplast DNA (cpDNA) studies (Aradhya *et al.*, 1999). Van Droogenbroeck *et al.* (2002), Jobin-Décor *et al.* (1997a,b) and Kim *et al.* (2002) confirmed the rehabilitation of the genus *Vasconcellea* (Badillo, 2000, 2001). Van Droogenbroeck *et al.* (2002) demonstrated that *Jacaratia* is more closely related to *Vasconcellea* than to *Carica*. *Vasconcellea* spp. have been studied *in situ* by Scheldeman *et al.* (2002) to evaluate the survival of populations for biodiversity conservation. These studies are critical for gene exploitation from these wild species (Drew *et al.*, 1998).

2.2.3. Fine mapping and physical mapping

Three hermaphrodite sex-linked markers, W11, T12 and CPBE55, have been used for fine mapping of the sex locus in 'Rainbow' F₂ and F₃ plants (Liu *et al.*, 2003, 2004). No recombination occurred between the markers and the sex locus. Southern hybridization of the sex markers with genomic DNA of staminate, hermaphrodite and pistillate plants showed that the markers are not present in the female. Sixty-two hermaphrodite sex-linked marker fragments have been cloned and sequenced to develop 42 SCAR markers. Twenty-seven of the markers have been used to screen the papaya BAC library. The physical distance between W11 and CPBE55 is 500 kb, 400 kb between CPBE55 and T12 and 900 kb between W11 and T12. Hybridizations of the aligned contiguous sequences (contigs) with the markers revealed numerous duplications in the genomic regions. A physical map was constructed of the suppressed region to characterize the genetic basis of sex determination (Liu *et al.*, 2004).

2.3. Marker-assisted selection

Eight enzyme systems have been used to identify molecular markers, but only limited polymorphisms existed in a small population of geographically diverse domesticated papayas (Manshardt, 1992). DNA polymorphisms using restriction fragment length polymorphism (RFLP) and RAPD fingerprinting with

11 inbred papaya cultivars and lines (Stiles *et al.*, 1993) revealed relationships among the papayas. More detailed RAPD data on papayas and relatives (Jobin-Décor *et al.*, 1997a,b; Magdalita *et al.*, 1997a, 1998) and AFLP data on 71 papayas and relatives showed similar relationships among the papayas (Kim *et al.*, 2002).

Marker-assisted selection in papaya is being attempted (R. Ming, personal communication). QTL phenotype data, e.g. earliness of flowering, height of flowering, stem diameter and tree height, have been collected in a segregating F₂ population from 'Rainbow'. A marker for the red flesh colour allele would be useful in back-crossing hybrids.

3. Micropropagation

Micropropagation of different genotypes has been reported (Mehdi and Hogan, 1976, 1979; Litz and Conover, 1977, 1978a,b, 1981a; Yie and Liaw, 1977; Pandey and Rajeevan 1983; Litz, 1984, 1986; Drew and Smith, 1986; Rajeevan and Pandey, 1986; De Winnaar, 1988; Drew, 1988; Miller and Drew, 1990; Reuveni *et al.*, 1990), and has been reviewed by Fitch (1995). Contamination of explants from field-grown trees is common. In general, Murashige and Skoog (1962) plant growth medium (MS) is supplemented with 1–10 µM of a cytokinin, e.g. kinetin or benzyladenine (BA), and 0.25–1 µM of an auxin, e.g. naphthaleneacetic acid (NAA), although Litz and Conover (1978a) used 50 µM kinetin and 10 µM NAA to induce growth in explants from mature field-grown plants and then substituted lower growth regulator concentrations for shoot proliferation. Rooting has been induced with 1–5 µM NAA and/or 0.5–20 µM indolebutyric acid (IBA). Drew and Smith (1986) showed that 10 µM riboflavin reduced callus growth during shoot proliferation, and obtained improved shoot quality with 1 µM each of NAA and BA.

Improved proliferation and rooting efficiency, reduced contamination and better acclimatization methods have been reported (Drew *et al.*, 1991, 1993, 1995; Drew, 1992; Yu *et al.*, 2000; Fitch, 2002; Fitch *et al.*, 2002b,c, 2003a,b). Drew *et al.* (1991) reported that

1 μM riboflavin together with 10 μM IBA resulted in 90% rooting. There have been other slight variations of protocol (Castillo *et al.*, 1997; Dam and Le, 1997; Schmildt *et al.*, 1997; Lai *et al.*, 1998; Bhattacharya *et al.*, 2001; Minh *et al.*, 2001b; Chan and Teo, 2002). Chan and Teo (2002) concluded that NAA was not essential for micropropagating 'Eksotika'.

Rooting medium consisting of half-strength MS in vermiculite coupled with the venting of vessels can improve plant growth *in vitro*, with increased survival *ex vitro* (Yu *et al.*, 2000). Difficult-to-detect bacteria that do not appear to inhibit shoot proliferation and elongation adversely affect root formation and root and shoot development in rooting medium containing sucrose (Fitch *et al.*, 2003a). Removal of sucrose from the rooting medium and indexing of stock cultures are important for controlling bacterial contamination (Fitch *et al.*, 2003a,b). Addition of 0.05–0.15% of a bacteriostatic mixture, PPMTM (0.135% of 5-chloro-2-methyl-3-(2H)-isothiazolone and 0.041% of 2-methyl-3-(2H)-isothiazolone, Phytotechnology, Inc., Kansas City, Missouri), to the rooting medium results in greener leaves and non-hyperhydric rooted plants.

Micropropagated plants in the field appear to be normal, with early and lower bearing of fruit and branching (Drew, 1988; Reuveni *et al.*, 1990; Fitch *et al.*, 2000, 2002; Fitch, 2002). Drew (1988) reported that flowers on micropropagated trees develop at 30–40 cm compared to typical seedlings, which set flowers at 150–200 cm. Drew (1992) and Drew *et al.* (1995) did not observe any morphological abnormalities in a field planting of 4000 micropropagated plants. Drew (1997) reported that 12,000 micropropagated plants were planted in a commercial field.

4. Somatic Cell Genetics

4.1. Regeneration

4.1.1. Somatic embryogenesis

Somatic embryogenesis of papaya was first described by DeBruijne *et al.* (1974), who induced somatic embryos from petiole sec-

tions on MS and White (1963) semi-solid media containing 0.001–10 μM auxins and cytokinins in a multistep protocol. Plants were not recovered from somatic embryos. Subsequently, Yie and Liaw (1977), Mehdi and Hogan (1979), Chen *et al.* (1987), Fitch and Manshardt (1990), Fitch (1993, 1995), Monmarson *et al.* (1994, 1995) and Mahon *et al.* (1996) described somatic embryogenesis and plant regeneration. Auxins, e.g. 6 μM or higher concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) or NAA, have generally been used.

Induction. Although embryogenic cultures have been induced from various explants in the presence of NAA (Yie and Liaw, 1977; Mehdi and Hogan, 1979; Chen *et al.*, 1987), the optimum protocol has involved zygotic embryos (Fitch and Manshardt, 1990; Cai *et al.*, 1999; Suksa-Ard *et al.*, 1999; Fernando *et al.*, 2001; Bhattacharya *et al.*, 2002) and seedling tissues, e.g. root tips, epicotyls, hypocotyls and cotyledons, in the presence of 2,4-D. Monmarson *et al.* (1994) induced embryogenic cultures from stem, epicotyl and petiole pieces of 2-month-old seedlings and from the integuments of 3- to 4-month-old immature seeds on medium with 9 μM 2,4-D (Monmarson *et al.*, 1995). The latter method enables the regeneration of maternal genotypes, although the percentage of explants that develop embryogenic cultures is low. Papaya can be regenerated from mature-phase material using petioles and root tip explants of micropropagated papayas (Yang and Ye, 1992; Yang *et al.*, 1996; Lin and Yang, 2001; Minh *et al.*, 2001a).

Maintenance. Mahon *et al.* (1996) initiated suspension cultures in medium containing 2 μM BA, 400 μM adenine sulphate and 0.5 μM NAA and maintained embryos in 0.5 μM BA and 0.05 μM NAA in suspension. Embryogenic cultures have been used for genetic transformation (Lines *et al.*, 2002). Suspension culture results in improved yields of somatic embryos and regeneration (Castillo *et al.*, 1998a).

Maturation. Addition of 0.5 μM abscisic acid (ABA) to semi-solid maturation

medium increases embryo size (Castillo *et al.*, 1998b). Castillo *et al.* (1998b) encapsulated somatic embryos in sodium alginate to develop artificial seeds. A 2.5% sodium alginate concentration in half-strength MS medium resulted in a high rate of germination, with normal plants.

Hybrid embryos resulting from crosses between PRSV-resistant wild species and papaya were rescued by culturing ovules, ovaries or dissected embryos because the seeds lacked endosperms (Litz and Conover, 1981a, 1982, 1983; Moore and Litz, 1984; Manshardt and Wenslaff, 1989a,b; Chen *et al.*, 1991; Magdalita *et al.*, 1996). The hybrid embryos were plated on modified MS medium with or without growth regulator supplements. Secondary embryos developed directly from 2-month-old zygotic embryos on growth regulator-free medium (Chen *et al.*, 1991). In an improved protocol, slightly older hybrid embryos, 3 to 4 months old, were pre-incubated for 5 days in liquid medium containing 10 μM gibberellic acid (GA_3), 0.25 μM BA and 0.25 μM NAA prior to transfer to growth regulator-free medium (Magdalita *et al.*, 1996).

Embryogenic cultures have also been established from related species, e.g. *V. stipulata* peduncles (Litz and Conover, 1980), *V. cundinamarcensis* hypocotyls (Jordan *et al.*, 1982) and *V. × heilbornii* nm. *pentagona* ovules (Vega de Rojas and Kitto, 1991).

Germination. Yie and Liaw (1977) reported germination of somatic embryos and recovery of normal plants (see Table 1 in Fitch, 1995). In most cases, auxin concentrations are reduced and cytokinin and gibberellin concentrations are increased.

4.1.2. Organogenesis

There have been relatively few reports of organogenesis with papaya (Arora and Singh, 1978; Litz *et al.*, 1983) and babaco (Jordan and Piwanski, 1997). Shoots have been regenerated from callus that developed on cultured seedling stem explants (Arora and Singh, 1978) and directly from cultured lamina and midribs of papaya cotyledons

(Litz *et al.*, 1983). Seedling stem tissues were grown on medium containing 11 μM NAA and 2.3 μM kinetin. The cotyledon cultures were grown on media containing 2.7–27 μM NAA and 1.3–8.9 μM BA. Young leaf explants of babaco developed shoots on medium containing 10 μM thidiazuron (Jordan and Piwanski, 1997).

4.1.3. Haploid recovery from anthers

Genetic stabilization of important traits has been traditionally achieved by selection and inbreeding for homozygosity. Chromosome doubling of haploids could result in immediate establishment of homozygosity for all loci. Recovery of papaya haploids from anther culture has been reported (Litz and Conover, 1978b; Tsay and Su, 1985), although culture conditions do not appear to have been optimized. Indeed, the latter study was repeated with 'Sunrise' and 'Rainbow' anthers without haploid production (T. Wenslaff, unpublished results). Manshardt (1992) commented that the heterozygous nature of hermaphrodite papaya could result in only females or lethal males.

4.1.4. Protoplast isolation and culture

Litz and Conover (1979) isolated protoplasts from papaya leaves with intentions of introgressing PRSV resistance genes from *V. cauliflora* and *V. stipulata* into papaya to overcome hybridization barriers to pollination. Isolation of protoplasts was also mentioned by Krishnamurthy *et al.* (1983). Jordan *et al.* (1986) fused protoplasts of papaya and *V. cundinamarcensis*, but plants were not recovered. Chen and Chen (1992) first regenerated plants from protoplasts prepared from embryogenic suspension cultures of papaya $\times$ *V. cauliflora* (Chen *et al.*, 1991). The digestion mixture consisted of Cellulase R-10 Macerozyme R-10 and Driselase in 0.4 M mannitol. Protoplast viability was 90%. Eight weeks after plating, torpedo-shaped embryos were observed. The regenerated plants were $2n = 18$ and appeared to be normal; they survived transfer to soil.

4.2. Genetic manipulation

4.2.1. Mutation induction and somaclonal variation

Naturally occurring papaya mutants have occasionally appeared in seedling populations (Storey, 1953; Nakasone and Paull, 1998): diminutive forms with small fruit and short petioles; plants with rugose leaves; red-fleshed fruit that is recessive to yellow; purple stems and petioles that are dominant to green; grey seed coat that is dominant to black, etc. The diminutive mutant gene is carried by line 55-1, the PRSV-resistant female R_0 transgenic 'Sunset' (Fitch *et al.*, 1992). Line 55-1 was outcrossed with a non-transgenic 'Sunset' (R. Manshardt, personal communication). β -1,3-Glucuronidase (GUS)-positive R_1 seedlings were selfed and successive generations were planted until the recessive mutant phenotype was eliminated in the R_4 generation. Apparently tetraploid plants with large, thick leaves and petals and small, usually sterile fruit were observed both in seedlings (M. Fitch, unpublished results; R. Manshardt, personal communication) and plants from *in vitro* cultures (Fitch, 2002; Fitch *et al.*, 2002c; M. Fitch, unpublished results). Females developed small, flat, miniature pumpkin-like parthenocarpic fruit, but they never formed fertile seed. Certain hermaphrodite trees occasionally set two to three seeds per fruit. Five seeds were grown and all reproduced similar thick-leaved plants that were similarly highly sterile.

4.2.2. Somatic hybridization

Breeding objectives. Although protocols have been developed for isolation, culture and regeneration from papaya and *C. papaya* $\times$ *V. cauliflora* hybrid embryogenic cultures (Chen and Chen, 1992), somatic hybrids have not been produced between papaya and the cold-tolerant, PRSV-resistant Andean species.

4.2.3. Genetic transformation

Breeding objectives. Genetic engineering for virus resistance has been an important goal, particularly for hermaphroditic papayas. Other pest problems have also been

targeted, e.g. fungal/oomycete and arthropod tolerance. Herbicide and aluminium resistance, improved shelf-life, increased firmness and cold tolerance are other important breeding objectives.

Neomycin phosphotransferase II (NPTII) and hygromycin phosphotransferase (HPT) genes, which confer antibiotic resistance, have been used for selection in most papaya transformation experiments. Three selection systems have been studied.

1. The mannose selection system (Joersbo *et al.*, 1998), using mannose rather than sucrose as a carbon source, avoids the use of antibiotic resistance; however, mannose at concentrations from 0.1 to 120 g/l, alone or together with sucrose, does not prevent secondary somatic embryo formation (Souza *et al.*, 2001).
2. The enzyme phosphinothricin acetyltransferase (PAT) or the bar gene imparts resistance to the herbicide phosphinothricin (Mohapatra *et al.*, 1999). Somatic embryos do not develop at phosphinothricin concentrations of 125 μ M or higher, and phosphinothricin-resistant transformed papayas can be recovered (Souza *et al.*, 2001).
3. Papayas have been transformed with a construct containing a non-destructive selection gene based on the green fluorescent protein (GFP) (Mankin and Thompson, 2001). A pBI121/GFP construct has been inserted into papaya (Zhu *et al.*, 2003c), and plants have been recovered.

Protocol. The transformation protocol for papaya (Fitch and Manshardt, 1990) has been based upon embryogenic cultures, following a method described for walnut (McGranahan *et al.*, 1988). Transformation has been achieved using either *Agrobacterium*- or particle gun-mediated gene transfer. The high success rate of transformation can be attributed, in part, to protocols that have been modified and improved to enhance transformation frequency, e.g. embryogenic suspension cultures (Lines *et al.*, 2002), agitation of embryogenic suspension cultures with carborundum particles and *Agrobacterium* (Cheng *et al.*, 1996; Yeh *et al.*, 1998), bombardment of thin layers of embryogenic cultures (Cai *et al.*, 1999),

comparison of kanamycin and geneticin for regeneration (Yu *et al.*, 2003) and somatic embryos in liquid culture wounded by vortexing with tungsten M-15 particles prior to co-cultivation with *Agrobacterium* (Ying *et al.*, 1999). Various other options have been described (Fitch *et al.*, 1994, 2002c; Fitch, 2002). A description of the plant growth media used in our laboratory is presented in Table 6.1.1, together with a brief description of our current transformation protocol. Promoters and selection genes used for the different transformation projects are included in Tables 6.1.2 and 6.1.3.

Mature papaya seeds, either freshly harvested or dried, are disinfested and agitated in sterile 1 M KNO₃ overnight, transferred to sterile water, and agitated at room temperature (26–28°C) until the testae crack. The seeds are germinated on 1% water agar. Hypocotyls from aseptic 10-day-old seedlings with newly unfolded cotyledons are sliced into 1 mm sections, plated on $\frac{1}{2}$ MSD medium and incubated at 26–28°C in the dark. After 6–8 weeks, embryogenic cul-

tures that develop from the slices are used for transformation.

Immediately following gene transfer, small clumps of embryogenic cultures and somatic embryos (2 mm diam.) are plated on $\frac{1}{2}$ MSD medium overlain with filter paper. Monthly subculturing is essential. The filter papers holding the clumps are transferred to selection medium, $\frac{1}{2}$ MSG50 and grown for 2 months. The selection stringency is increased by transfer to $\frac{1}{2}$ MSG100 for 2 months or until selectively growing cultures appear. Elimination of escapes can be achieved by culturing selectively growing lines on $\frac{1}{2}$ MSG300 for 1–2 months. Selected lines are regenerated on MBN medium. Shoots 0.5–5.0 cm long are induced to root on medium containing IBA in the dark for 5–30 days, and then transferred to VM medium for root development. Following acclimatization, plants can be potted in a peat/perlite mixture, grown under clear plastic domes for 1–2 weeks, transferred to the greenhouse for 1–2 weeks, acclimatized in sunlight for 1 week and planted in the

Table 6.1.1. Media used for tissue culture in papaya transformation, Hawaii.

Name of medium	Contents and methods
MS	Murashige and Skoog (1962) medium, major and minor salts + 100 mg/l myo-inositol, 4 mg/l thiamine HCl, pH 5.8
MSO	MS + 3% sucrose + 2.5 g/l Phytagel
Somatic embryo proliferation medium = $\frac{1}{2}$ MSD	50% MS + 7% sucrose + 10 mg/l (45.5 μ M) 2,4-D + 2.5 g/l Phytagel, pH 5.8
Selection media = (1) $\frac{1}{2}$ MSG50 (2) $\frac{1}{2}$ MSG100 (3) $\frac{1}{2}$ MSG300	1 = $\frac{1}{2}$ MSD + 50 mg/l geneticin, 2 = $\frac{1}{2}$ MSD + 100 mg/l geneticin, 3 = $\frac{1}{2}$ MSD + 300 mg/l geneticin, pH 5.8
Regeneration medium = MBN	MSO + 0.2 mg/l (1.1 μ M) naphthaleneacetic acid (NAA) + 0.2 mg/l (0.89 μ M) benzyladenine (BA). Selected calluses cultured on MBN for 3–6 months or until shoots developed, shoots transferred to rooting medium
Root induction medium = IBA	MSO + 2 mg/l (9.8 μ M) indolebutyric acid (IBA). Shoots incubated on MS/IBA for 5 days in the dark
Rooting medium = VM	Jars of vermiculite moistened with $\frac{1}{2}$ MS + 3% sucrose, v/v = 45% vermiculite/55% liquid medium. Shoots from MS/IBA transferred to VM and grown in bright light until roots were observed. Vented lids replaced solid lids for 1–2 weeks. Shoots transplanted to potting mixture (peat/perlite) and acclimatized under clear plastic domes, vented over 1–2 weeks, transferred to the greenhouse for about 1 month and to full sunshine for 1–2 weeks prior to field planting

Table 6.1.2. Groups involved in papaya transformation for virus resistance worldwide.

Papaya problem	Group	Location	Construct	Results	Reference
PRSV	L. Herrera-Estrella	Mexico	CP, 35S promoter, NPTII	Greenhouse tests, field test moratorium	Personal communication
PRSV	P. Tennant, D. Gonsalves	Jamaica	CP, UT, 35S promoter, NPTII selection	Resistance: field tests, final stages of commercialization	Tennant <i>et al.</i> , 2002; personal communication
PRSV	M. Davis	Florida	CP, UT, 35S promoter, NPTII selection	Tolerance: R ₂ field tests, R ₃ seed from UT	Davis and Ying, 2002; personal communication
PRSV	J. Dale, M. Bateson, R. Drew, D. Persley, R. Lines	Australia	CP, UT, 35S promoter, NPTII selection	Resistance: field tests	Mahon <i>et al.</i> , 1996; Lines, 2002; Lines <i>et al.</i> , 2002
PRSV	M. Souza, D. Gonsalves	Brazil	CP, UT, 35S promoter, NPTII selection	Resistance: greenhouse tests, no field test permit yet	Souza, 1999; personal communication
PRSV	N. Sarindu, V. Prasartsee, D. Gonsalves	Thailand	CP, UT, 35S promoter, NPTII selection	Resistance: field tests	Gonsalves, 1998
PRSV	P. Magdalita	Philippines	CP, 35S promoter, NPTII selection	Resistance: greenhouse tests	Personal communication
PRSV	Y.K. Chan, V. Pilasina	Malaysia	CP, UT, 35S promoter, NPTII selection	Greenhouse: field tests	Personal communication
PRSV	A. Supat, S. Chowpongpan	Thailand	CP, 35S promoter, NPTII selection	Resistance: field tests	Personal communication (K. Romyanon)
PRSV	G. Chen	China	Replicase, 35S promoter, NPTII selection	Resistance: field tests	Chen <i>et al.</i> , 2001
PRSV	T. Zimmerman	US Virgin Islands	CP gene, 35S promoter, NPTII selection	Resistance: R ₁ field tests, R ₂ planted	Zimmerman and St Brice, 2003; personal communication
PRSV	D. Gonsalves, R. Manshardt, M. Fitch, J. Slightom	Hawaii	CP, 35S promoter, NPTII selection	Immunity: commercialized cultivars	Fitch <i>et al.</i> , 1992; Lius <i>et al.</i> , 1997; Gonsalves, 1998; Ferreira <i>et al.</i> , 2002; Fitch, 2002; Fitch <i>et al.</i> , 2002a,b,c
PRSV-Kapoho	D. Gonsalves, S. Ferreira	New York, Hawaii	CP, UT, multiCP, 35S promoter, NPTII selection	Resistance: R ₀ field tests, R ₁ seeds	Personal communication
PRSV-Kamiya	M. Fitch, T. Leong	Hawaii	CP, UT, replicase, 35S promoter, NPTII selection	Immunity (UT), resistance (CP, replicase): R ₀ , R ₁ field tests, R ₂ seeds	Fitch <i>et al.</i> , 1998; Fitch, 2002; Fitch <i>et al.</i> , 2002a; T. Leong, unpublished results
PRSV, PLDMV	S.-D. Yeh	Taiwan	CP, UT, 35S promoter, NPTII selection	Broad-spectrum immunity, resistance: completed PRSV field tests, PLDMV field tests	Yeh and Bau, 2001; Chen <i>et al.</i> , 2002; Yeh <i>et al.</i> , 2002; Bau <i>et al.</i> , 2003;

PRSV, papaya ringspot virus; PLDMV, papaya leaf distortion mosaic virus; CP, coat protein gene; UT, untranslatable coat protein gene; multiCP, multiple coat protein genes.

Table 6.1.3. Groups transforming papaya for other input traits.

Papaya problem	Group	Location	Construct	Results	Reference
PRSV, PLDMV	T. Maoka, K. Ogawa	Japan	CP, UT, multiCP, 35S promoter, NPTII selection	Tissue culture, greenhouse	Personal communication
Fungi	M. Fitch, J. Zhu, S. Ferreira	Hawaii	Chitinases from rice, <i>Streptomyces albolflavidus</i> , <i>Manduca sexta</i> ; stilbene synthase; defensins (lipid transfer proteins); 35S promoter, wound inducible promoter, NPTII, HPT selection	Tissue culture, greenhouse, field tests	Zhu <i>et al.</i> , 1999, 2001a,b, 2002b, 2003a,b; J. Zhu, M. Fitch, unpublished results
Fungi	J. Zhu	Hawaii	Pathogenesis-related protein controller gene (NPR1); AP24 gene; PR1 gene	Tissue culture, greenhouse, field tests	Personal communication
Fungi	S.-D. Yeh	Taiwan	Rice chitinase, 35S promoter, NPTII selection	Greenhouse tests	Personal communication
Fungi	M. Souza	Brazil	Magainins, 35S promoter, NPTII selection	No resistance to <i>Asperisporium caricae</i>	Personal communication
Arthropods	J. Zhu, H. McCafferty, M. Fitch	Hawaii	Chitinases from <i>Manduca sexta</i> , <i>Streptomyces albolflavidus</i> ; lectin from <i>Galanthus nivea</i> , rabisco small subunit promoter, NPTII selection	Tissue culture, greenhouse, field tests	McCafferty <i>et al.</i> , 2003; personal communication; M. Fitch, unpublished results
Delayed ripening	S. Attatom, K. Romyanon	Thailand	Antisense ACC synthase, 35S promoter, NPTII selection	Greenhouse tests	Personal communication
Delayed ripening	P. Magdalita, A. Laurena	Philippines	Antisense ACC synthase and oxidase, 35S promoter, NPTII selection	Greenhouse tests	Laurena <i>et al.</i> , 2002; Magdalita <i>et al.</i> , 2002a,b; personal communication
Delayed ripening	J. Botella	Australia	Antisense and sense ACC synthase, ACC synthase RNAi, antisense <i>etr1-1</i> with a fruit-specific promoter, 35S promoter, NPTII selection	Tissue culture, greenhouse, field tests	Personal communication
Delayed ripening	Y.K. Chan, U. Bakkar	Malaysia	Antisense ACC synthase and oxidases, 35S promoter, NPTII selection	Field tests	Personal communication
Delayed ripening	J. Stiles, K. Neupane	Hawaii	Antisense ACC synthase and oxidase, 35S promoter, NPTII selection	Field tests: no delay in hemizygous lines	Neupane, 1997; personal communication
Delayed ripening	S. Ferreira, D. Gonsalves	Hawaii	Antisense polygalacturonidase, 35S promoter, NPTII selection	Tissue culture, greenhouse	Personal communication
Increased firmness	R. Paull, N. Chen	Hawaii	Antisense endoxylanase, 35S or sugarcane ubiquitin promoter, NPTII selection	Greenhouse tests	Chen and Paull, 2003; personal communication

Continued

Table 6.1.3. *Continued.*

Papaya problem	Group	Location	Construct	Results	Reference
Herbicide resistance	L. Herrera-Estrella	Mexico	Acetolactate synthase mutant, 35S promoter, NPTII, PPT selection	Greenhouse tests: resistance	Cabrera-Ponce <i>et al.</i> , 1995
Resistance to aluminium toxicity	L. Herrera-Estrella	Mexico	Over-expression of citrate synthase, 35S promoter, NPTII selection	Greenhouse tests: resistance	de la Fuente <i>et al.</i> , 1997
Cold tolerance	R. Litz, S. Dhekney, A. Yadav	Florida	Cold tolerance gene ^a from <i>V. cundinamarcensis</i> , 35S promoter, NPTII selection	Tissue culture	S. Dhekney, R. Litz, personal communication
Alternative selection systems	M. Souza	Brazil	Mannose and phosphinothricin resistance genes (PMI and PAT)	PMI not suitable; PAT possibly useful	Souza <i>et al.</i> , 2001
Alternative selection systems	J. Zhu, R. Agbayani	Hawaii	PMI and green fluorescent protein genes	Greenhouse plants: both protocols feasible	Zhu <i>et al.</i> , 2003c; personal communication

^aCold tolerance gene, C-repeat/dehydration-responsive element binding factor, CBF (Jaglo-Kirsten *et al.*, 2001).

ACC, aminocyclopropane carboxylase; RNAi, interfering RNA transformation to 'knock out' gene function (Hannon, 2002); etr1-I, a mutated receptor gene of *Arabidopsis* that confers dominant ethylene insensitivity (Wilkenson *et al.*, 1997); PMI, phosphomannose isomerase (Joersbo *et al.*, 1998); PAT, phosphinothricin acetyl transferase (Mohapatra *et al.*, 1999); PPT, phosphinothricin.

field. Transformation efficiency is approx. 12 different transgenic lines from each bombarded plate. Transgenic plants can be recovered after 6–9 months, and approx. 15% of the transgenic lines are aberrant, either apparent tetraploids or variants that cannot be rooted.

Accomplishments

PRSV resistance. Although conventional breeding for enhancing tolerance of PRSV and cross-protection of papaya are appropriate strategies for some regions, breeding problems of hermaphroditic papayas can be more readily addressed by genetic transformation. Pang and Sanford (1988) reported the recovery of transgenic papaya callus following *Agrobacterium* infection of leaf discs; however, plants were not regenerated. The first genetically engineered papaya plants expressed the *uidA* (β -glucuronidase, GUS) and *nptII* (neomycin phosphotransferase II) transgenes and were kanamycin-resistant (Fitch *et al.*, 1990). Papaya was transformed

with a construct containing the PRSV coat protein gene of a mild cross-protecting strain, HA5-1, from Hawaii (Fitch *et al.*, 1990; Ling *et al.*, 1991). A few lines were PRSV-resistant (Fitch *et al.*, 1992; Tennant *et al.*, 1994; Lius *et al.*, 1997). One R₀ line, 55-1, a red-fleshed 'Sunset' female, containing a single transgene insertion site, was outcrossed with non-transgenic 'Sunset' and the GUS-positive R₁ progeny were self-pollinated. Homozygous R₂ individuals were identified by GUS expression in R₃ seeds (R. Manshardt, unpublished results). Homozygous R₄ plants were designated 'SunUp' and crossed with non-transgenic 'Kapoho', the yellow-fleshed Hawaiian industry standard. The resulting hybrid, 'Rainbow' (Manshardt, 1998), was yellow-fleshed since the yellow colour allele is dominant.

'SunUp' was immune to the Hawaiian strain of PRSV; however, it showed no resistance to PRSV strains from Thailand and Taiwan, both of which showed the greatest base pair divergence from the Hawaiian iso-

late (Tennant, 1996; Tennant *et al.*, 2001). Another immune line, 63-1, apparently contained two unlinked copies of the PRSV resistance gene and in the homozygous state was immune to all strains screened (Tennant *et al.*, 2001). In the field, hemizygous 'Rainbow' seedlings were susceptible to Hawaiian PRSV for up to 77 days or until the seedlings became established (Gaskill *et al.*, 2002). Thereafter, plants could succumb to PRSV at rates of about 1/700 to 1/1000 (M. Fitch, unpublished results). The rationale was that post-transcriptional gene silencing, the apparent mode of resistance in 'Rainbow' and its progeny (Tennant *et al.*, 2001), was not fully effective in young seedlings and propagules in the hemizygous state. Two-month-old micropropagated 'Rainbow' grown under fluorescent lights in the laboratory were 100% infected by hand inoculation of PRSV (M. Fitch, unpublished results). Line 55-1, which is a hemizygous R_0 transgenic line, never succumbed to PRSV after multiple hand inoculations in the greenhouse (Fitch *et al.*, 1992).

'SunUp' and 'Rainbow' are the first genetically engineered, PRSV-resistant papayas, and are the first fruits deregulated and licensed for commercialization in the USA. Release of transgenic papayas was timely. In 1992, PRSV was discovered in the previously pristine papaya-growing region of Hawaii. After R_0 line 55-1 was shown to be PRSV-resistant in the greenhouse, 6 years were required to achieve deregulation and 7 years were needed to license intellectual properties. The papaya industry and the University of Hawaii created an educational video, devised a distribution plan and provided the seeds free of charge to farmers impacted by the disease.

Five years after commercialization of plants with the improved input trait, the Hawaiian papaya industry rebounded, although overproduction, recapturing markets lost to PRSV, implementing higher fruit quality standards and the controversy over the use of its genetically modified (GM) fruit continue to be problems. Corporations have banned GM papaya, due to public perception of product safety. Although Canada approved import of GM papayas in 2003,

other foreign export markets remain closed. The papaya is a model to demonstrate the attributes of GM foods. None the less, alternatives to genetic transformation are being re-examined, e.g. cross-protection, wide hybridizations and multigenic tolerance, because many markets and consumers continue to resist adoption of GM papayas. Genomics could be used to fingerprint the tolerance phenotype and identify it in linkage-mapped segregating populations (Kim *et al.*, 2002; Ma, 2003).

Back-crossing and selection. 'SunUp' and 'Rainbow' have been hybridized with other important Hawaiian cultivars (Fitch *et al.*, 2002b). The industry standard, 'Kapoho', a small-fruited (c. 460 g) papaya with firm yellow flesh, was the preferred export cultivar prior to the release of 'Rainbow' and is still the most important non-transgenic export cultivar. The large-fruited 'Kamiya' (c. 750 g) often occupies the highest price niche on the local market, commanding farm-gate prices two to four times greater than those of 'Kapoho' or 'Rainbow'. Back-cross hybridization of 'Kapoho' and 'Kamiya' with 'Rainbow' F_2 plants, followed by selection for quality characteristics, has been used to introgress PRSV resistance (Fitch *et al.*, 2001a,b). 'Laie Gold', a hybrid of 'Kamiya' and transgenic 'Rainbow' F_2 plants, has been under evaluation since 1999 (Fig. 6.1.2). Although 'Laie Gold' is an excellent cultivar, consumers have differentiated its flavour and other characteristics from those of 'Kamiya'. While transgenic 'Kamiya' back-cross₃ (BC_3) and BC_4 fruit resemble the inbred line more closely, after 4 years of comparisons, the hybrid vigour of the F_1 over back-crosses makes the former a preferred cultivar. Currently, clonally propagated 'Laie Gold' and 'Kamiya' BC_1 selections are being evaluated in three different locations in order to identify selections with high yield and quality (Fitch *et al.*, 2002a).

'Rainbow' papaya, a yellow-fleshed cultivar, has become a dominant cultivar due to its PRSV resistance. Growers, however, prefer smaller, firmer-fleshed yellow fruit with the good shipping qualities of 'Kapoho'. Therefore, 'Rainbow' F_2 seedlings have been

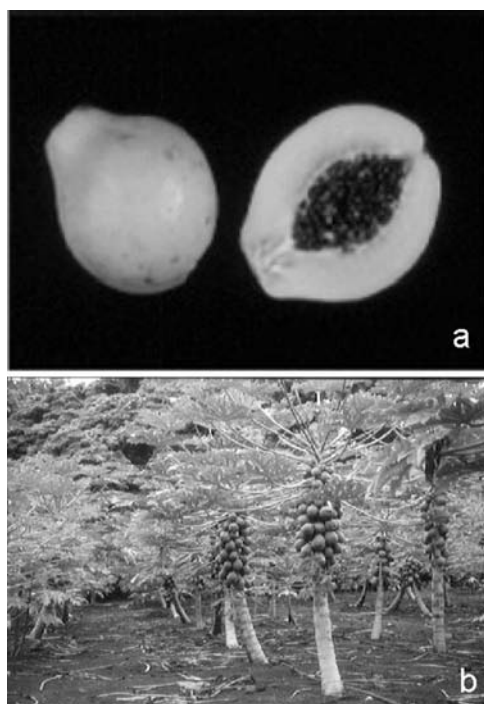


Fig. 6.1.2. 'Laie Gold', fruit (a) and tree (b), a virus-resistant F_1 hybrid between 'Kamiya' and 'Rainbow' F_2 .

back-crossed with 'Kapoho'. A pleasing 'Kapoho' BC_1 was selected and named 'Poamoho Gold'; fruit size is nearly the same as 'Rainbow'. Its flesh colour is deep orange-yellow like 'Rainbow', but segregation of firmness and size was unexpected after two rounds of back-crossing. 'Kapoho' BC_3 and BC_4 fruits were smaller than BC_2 . Hermaphrodite seedlings of self-pollinated 'Kapoho' BC_4 have been micropropagated and are under evaluation. 'Rainbow' F_2 lines that are homozygous for yellow flesh colour and PRSV resistance have been distributed to cooperating growers, who are recurrently selecting lines having good fruit quality. Fruit size in F_5 plants has decreased due to inbreeding depression. One grower's F_6 seedlings segregated for fruit size, and selections for a certain fruit size were made. The F_6 inbreds showed decreased hybrid vigour compared to the 'Rainbow' F_1 .

A few Hawaiian cultivars are grown for speciality purposes, e.g. green papayas for

salad and for cooking. Growers in high-rainfall areas have selected for higher root rot-tolerant lines. F_1 hybrids between 'KL Yellow' and 'Harold' and a transgenic 'Rainbow' F_2 with good-quality fruit were intermediate in size and shape between the two parental inbred lines. F_1 hybrids between a large-fruited Thai inbred, 'Khakdum', and a transgenic 'Rainbow' F_2 and between a 'Kamiya'/'Rainbow' back-cross resulted in F_1 fruit with the distinct flavour of 'Khakdum'. Fruit of F_1 hybrids between 'Rainbow' F_2 and a high-rainfall, soil-adapted, large-fruited inbred line, 'Maunawili Sweet', were large, and the BC_2 hybrids were micropropagated for testing along with a selected micropropagated F_1 line and the inbred line. The Australian dioecious 'Drew', with blemish-free skin and leafhopper tolerance but an unpleasant flavour, has been crossed with 'SunUp' and produced large hybrid fruit with fairly blemish-free skin, but the fruit texture and flavour remain unacceptable (F. Zee, personal communication).

In 2003, there were approximately ten different groups who were active in papaya transformation for virus resistance (Table 6.1.2). Transgenic resistance to a second papaya virus, papaya leaf distortion mosaic virus (PLDMV), is being pursued (Lai *et al.*, 2002; T. Maoka, unpublished results). Since PRSV resistance is specific for the virus strain from which the resistance cassette was constructed (Tennant *et al.*, 2001; Bau *et al.*, 2003), each group has constructed vectors with resistance genes from local PRSV strains and transformed locally important cultivars. In Taiwan, PRSV-resistant lines have been field-tested for > 6 years (Yeh *et al.*, 1998; Bau *et al.*, 2003), but the GM controversy has impeded commercialization. Unlike the narrow resistance of the Hawaiian transgenic papayas (Fitch *et al.*, 1992; Tennant *et al.*, 1994, 2001), some of the Taiwanese transgenic papayas are immune to Taiwan, Thai, Hawaiian and Mexican strains of PRSV, the first report of such broad-spectrum immunity for viruses differing in coat protein (CP) gene sequences up to 89% (Bau *et al.*, 2003). Bau *et al.* (2003) recovered a larger number of transgenic lines and

their construct was larger, including 206 nucleotides (nt) vs. 49 for Hawaii. The 3' region of a transgene is often the target of post-transcriptional gene silencing. Bau *et al.* (2003) believed that the highly conserved regions of the CP gene and the 3' or the highly conserved regions of other PRSV genes, e.g. the RNA replicase domains of the NIb gene, can be used to generate broad-spectrum resistance mediated by RNA silencing. They contend that their success is because a transgene construct containing the complete coding region of the CP gene and the 3' non-coding regions for a potyvirus coupled with screening from large numbers of transgenic plants can provide a broader spectrum of resistance against different strains. The Taiwan papayas appear to have general usefulness in other papaya-growing areas if PRSV is the only viral pathogen. The PRSV transformants appear to be very susceptible to infection by PLDMV (Yeh and Bau, 2001), a potyvirus with symptoms nearly identical to those of PRSV. PLDMV has been reported in Okinawa, Taiwan and Saipan (Maoka *et al.*, 1996, 1997; Yeh and Bau, 2001; Yeh *et al.*, 2002). Papaya has been transformed with both PRSV and PLDMV CP genes (Yeh *et al.*, 2002; T. Maoka, unpublished results), and resistance to the two viruses was demonstrated (Yeh *et al.*, 2002).

Plants transformed with a multiple-strain construct containing fragments of the CP gene from Thai, Taiwanese and Hawaiian strains of PRSV have resistance to all strains (Table 6.1.2). In Hawaii, 'Kamiya' was transformed with a translatable or untranslatable CP gene or a translatable replicase gene (Table 6.1.2). One line with the untranslatable construct contained about eight copies of the transgene and never showed symptoms of infection after more than five PRSV inoculations. R₁ seedlings were screened by Southern hybridization to select homozygous, multiple-copy lines for further characterization. Translatable CP- and replicase-transformed lines were mostly aberrant plants, probably tetraploids, highly PRSV-resistant but sterile. One replicase-transgenic line was immune and appeared to be normal.

Virus resistance in transformants is being

evaluated in greenhouse or field tests in the Philippines, Malaysia, China (Chen *et al.*, 2001), Australia (Lines *et al.*, 2002), Jamaica and Thailand (Table 6.1.2). Controversy over GM foods has resulted in a field test moratorium in Mexico and Brazil, and PRSV-resistant lines remain in greenhouses. Brazilian scientists have begun to characterize a new papaya virus with a 12 kb double-stranded genome (M. Souza, personal communication). Engineered resistance could be utilized if genetic resistance cannot be found.

Transformation with genes for other traits.

Table 6.1.3 lists other priorities for transforming papaya, including fungal and arthropod resistance, delayed ripening and herbicide and aluminium tolerance.

Fungal disease resistance has been difficult to engineer because resistance assays are less precise and a broad array of fungi cause plant and fruit diseases. M.T. Souza (personal communication) determined that magainin (Zasloff, 1987) is ineffective for attenuating black spot. Furthermore, use of a frog gene in papayas could present public acceptance problems. Transformation of papaya with a variety of anti-fungal genes has been reported (Zhu *et al.*, 1999, 2001a,b, 2002b, 2003a,b; J. Zhu, M. Fitch and S. Ferreira, unpublished results). Strong evidence for resistance, however, has not been established. The anti-fungal genes utilized in these studies include rice chitinase (Lin *et al.*, 1995; S.D. Yeh, personal communication) from S. Muthukrishnan (Kansas State University), *Streptomyces albidoflavus* chitinase (Broadway *et al.*, 1995) from D. Gonsalves (Cornell University), anti-microbial peptides of dahlia, radish and onion (Terras *et al.*, 1992a,b, 1995; Cammue *et al.*, 1995), stilbene synthase (Hain *et al.*, 1993), a PR protein controller gene, NPR1 (Cao *et al.*, 1998), *Manduca* chitinase (Ding *et al.*, 1998) and citrus AP24, an osmotin PR protein from R. Maier (US Department of Agriculture) (USDA).

Transgenic papayas expressing a rice chitinase gene (Lin *et al.*, 1995) inhibited growth of *P. palmivora* mycelia in an *in vitro* assay and larger numbers of transgenic plants compared to controls survived root

drench assays (Zhu *et al.*, 1999, 2002b). Rice chitinase transformants developed fewer powdery mildew pustules compared to known susceptible lines (M. Fitch, unpublished results). Some lines transformed with the *Dahlia merckii* anti-fungal peptide or stilbene synthase construct showed some root drench tolerance of *Phytophthora* (Zhu *et al.*, 2001a,b, 2002b, 2003a,b; J. Zhu, unpublished results). Papaya leaf discs from plants transformed with the *Arabidopsis* NPR1 gene exhibited fewer anthracnose lesions than controls and rice chitinase- and stilbene synthase-transformed plants (J. Zhu, unpublished results).

Infested fruit are banned from importation into the US mainland and Japan. Other insect and acarid pests of papaya attack the leaves and fruit, lowering quality and productivity. The Steven's leafhopper, *E. stevensi* Young, causes leaf chlorosis and inhibits leaf enlargement. Three species of mites attack papaya leaves, causing chlorosis (*C. brionese*, *T. cinnabarinus*) and deformity (*B. phoenicis*). The genes introduced into papaya for arthropod (and fungal/oomycete) resistance include *Streptomyces* chitinases (Broadway *et al.*, 1995, 1998; Gongora, 1999; Gongora *et al.*, 2001), *Manduca sexta* chitinase (Ding *et al.*, 1998), described for fungal resistance, and snowdrop lectin (Bell *et al.*, 1999). The *M. sexta* chitinase gene increased tolerance of *T. cinnabarinus*, but, after non-transgenic plants were killed, the population migrated to the transgenic plants (McCafferty *et al.*, 2003; H. McCafferty, unpublished results). Plants transformed with bacterial chitinase are being grown in the greenhouse for bioassays and R₁ seed production.

Papaya is a climacteric fruit and ripening is accelerated in closed shipping containers (Nakasone and Paull, 1998). Losses could be avoided by shipping fruit that generate low levels of ethylene. Shelf-life of fruit can also be extended if ethylene production is inhibited, e.g. by DNA antisense, co-suppression and/or RNA interference (RNAi) technology. Neupane (1997; Neupane *et al.*, 1997) transformed papaya for delayed ripening using ACC synthase and ACC oxidase genes from papaya; however, delayed ripening and decreased fruit softening were not observed

(K. Neupane, personal communication). The transgenic plants were self-pollinated to determine if the transgenes in the homozygous condition would impart some delay in ripening, but no delay was observed (K. Neupane, unpublished results). In a similar study, papaya has been transformed with antisense and sense ACC synthase from 'Sunrise' papaya (J. Botella, University of Queensland, Australia, unpublished results).

'Sunrise' papayas have been transformed with ACC synthase RNAi (Hannon, 2002) and the mutant receptor gene from *Arabidopsis* *etr1-1* (Wilkinson *et al.*, 1997), driven by a ripening-specific promoter to control ethylene production. The *etr1-1* gene confers ethylene insensitivity in plants, but tobacco plants transformed with *etr1-1* are susceptible to fungal pathogens (Geraats *et al.*, 2003). Attempts are being made to extend and control papaya shelf-life by controlling ethylene production, using antisense ACC synthase and antisense polygalacturonidase transformation (Sheehy *et al.*, 1988). The antisense xylanase gene has been introduced into papaya to reduce fruit softening (N. Chen and R. Paull, personal communication).

Cabrera-Ponce *et al.* (1995) reportedly developed herbicide-tolerant papaya after transformation with PAT. Selection was accomplished using both kanamycin and phosphinothricin, and transformed plants were resistant to phosphinothricin.

De la Fuente *et al.* (1997) transformed papaya with citrate synthase to confer tolerance of aluminium toxicity. Transgenic plants that over-produced citrate synthase could theoretically be grown in marginal lands of highly weathered, aluminium-bearing soils and bound phosphate could be released from these soils for plant nutrition. Delhaize *et al.* (2001), however, repeated this study with tobacco and could not confirm these results. Due to the moratorium on field testing in Mexico, the aluminium-tolerant plants remain in the greenhouse (L. Herrera-Estrella, personal communication).

Papayas grow in a fairly narrow subtropical climatic zone between 23°N and S lati-

tudes, although growers have planted in the temperate zone up to 32°N and S (Nakasone and Paull, 1998). The mountain papaya, *V. cundinamarcensis* (formerly *Carica pubescens*, Badillo, 2000), grows at elevations of 1000 m or greater in the Andes (Jordan *et al.*, 1982, 1986; Scheldeman *et al.*, 2002). Genes for cold tolerance were sought using a heterologous probe from tomato (Jaglo-Kirsten *et al.*, 2001). The homologue from papaya was cloned and is being transformed into papaya, using *Agrobacterium*, and cultures are being regenerated (R. Litz and S. Dhekney, personal communication).

4.3. Cryopreservation

Papaya has been conserved for short terms, mainly as refrigerated dried seeds and, to a lesser extent, as micropropagules *in vitro*. Clonally propagated cultivars, especially selections and complex hybrids, cannot be conserved as seeds. Rooted cuttings or micropropagation alternatives require space, time and resources for maintenance. Seed viability deteriorates after 5 years (Ellis *et al.*, 1991; Wood *et al.*, 2000). Therefore, long-term storage by cryopreservation (Chin and Krishnapillay, 1989; Althoff and Carmona, 1999; Ashmore *et al.*, 2001; Azimi *et al.*, 2001) has been a priority. Cryopreservation could be used for germplasm conservation and for storage of embryogenic cultures and somatic embryos required for micropropagation, genetic transformation and selection studies. Medium-term storage of papaya shoot tips *in vitro* for 1 year, using 1% fructose as a substitute for 2–3% sucrose, has been reported (Drew, 1988; Lai *et al.*, 2002). Plants can survive on medium supplemented with 1% fructose, with or without growth regulators, while those on sucrose-containing media died.

4.3.1. Seeds and shoot tips

Seeds and embryos of papaya desiccated to < 10% moisture content and stored in liquid nitrogen at –196°C for 24 h remain viable, although the rate of germination and seedling growth rate are reduced (Chin and

Krishnapillay, 1989). Althoff and Carmona (1999) desiccated seeds to 5.3–9.8% moisture content, packaged them in foil and immersed them in liquid nitrogen. Following slow thawing, seeds remained viable. Seeds and shoot tips of papaya have been cryopreserved (Azimi *et al.*, 2001). Seeds desiccated to moisture contents of 40–5% fresh weight consistently showed 70–90% germination rates. Desiccated seeds were frozen in liquid nitrogen for 30 min, thawed and germinated. The germination rate of seeds at 100%, 40%, 20% and 15% moisture content was 0, 8, 6 and 8%, respectively. The highest germination rate of 48% was achieved at 10% moisture content, followed by 20% germination for seeds stored at 5% moisture content. The growth rate of seedlings from cryopreserved seeds measured 10 weeks after planting was reduced to 25% that of controls.

Cryopreservation of three genotypes and regeneration of plants from > 65% of the vitrified/cryopreserved shoot tips have been reported (Ashmore *et al.*, 2001; Azimi *et al.*, 2001). Shoot tips from micropropagated plants were incubated in liquid medium (DeFossard *et al.*, 1974) for 1–6 days prior to vitrification in several concentrations of plant vitrification solution 2 (PVS2) medium (Sakai *et al.*, 1991). The optimum time for 70% survival and regeneration was 4 days after exposure to 100% PVS2 for 20 min. No regeneration occurred following incubation in PVS2 for < 20 min or > 40 min and liquid nitrogen treatment. After thawing, shoot tips were rooted for field evaluation. Cryopreserved shoots showed significantly reduced growth rate. After 2.5 years in the field, the plants were < 1 m in height. The effect of cryopreservation on seed and shoot tip growth reduction requires further investigation.

4.3.2. Embryogenic cultures and somatic embryos

Lu and Takagi (2000) cryopreserved somatic embryos of ‘Sunrise’ papaya by the vitrification method. Small clusters of adventitious embryos smaller than the torpedo stage were treated with a mixture of 1.5 M glycerol

erol, 0.4 M sucrose and 5% dimethyl sulphoxide (DMSO) for 25 min at 25°C, dehydrated with PVS2 for 25 min and plunged into liquid nitrogen. Vitrified and thawed embryos resumed growth within 1 week. Embryos matured without forming secondary embryos. More than 70% of the embryos germinated and grew into plants. Several cryoprotectant combinations were tested by Dhekney *et al.* (2003): (i) glycerol + DMSO, 5% each; (ii) glycerol + DMSO, 10% each; (iii) polyethylene glycol + glucose + DMSO, 10% each; and (iv) glycerol 30% + ethylene glycol 15% + DMSO 15%. Embryogenic cultures were incubated in 1.5 ml cryovials, cooled at 1°C/min to -80°C and plunged into liquid nitrogen. Viability and growth of cultures were recorded after 48 h in storage. The highest survival immediately after freezing (100%) occurred with the treatment consisting of 10% each of glycerol and DMSO. After 2 months, viability was highest with the vitrification procedure, and somatic embryo development was observed from these cultures. Long-term growth data were not presented in either study.

5. Conclusions

Biotechnology saved the Hawaiian papaya industry and underscores the potential for this new science. Hawaii, with relief from virus destruction, now has the opportunity to develop and market the highest quality fruit as other pest problems receive atten-

tion. Transformation for pest and pathogen resistance, improved shelf-life and herbicide and aluminium tolerance could lead to further improvements of this crop. Genomics present the potential for the discovery of genes to help plant breeders produce improved cultivars and germplasm when markers are identified for horticulturally important traits. Large numbers of markers have been generated by genomics; the task at hand is to correlate traits with the markers.

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7

Clusiaceae

The *Clusiaceae* or *Guttiferae* are natives of the New and Old World tropics and include several well-known species, e.g. the signature tree (*Clusia rosea* Jacq.), mamee apple (*Mammea americana* L.), ironwood tree (*Mesua ferrea* L.) and Maria (*Calophyllum brasiliense* Camb.). There are approx. 40 genera containing approx. 1000 species (Watson and Dallwitz, 1992 onwards). The genus *Garcinia* is composed of at least 39 species, and probably originated in the Malaysian Archipelago (Lim, 1984). There are many fruit-bearing *Garcinia* species, but none are as popular as the mangosteen (*Garcinia mangostana* L.). The *Garcinia* species have not spread widely from their centre(s) of origin due to the recalcitrant nature of the seeds, which quickly lose their viability. Corner (1952) reported that the mangosteen was probably domesticated in Thailand or Burma. Other related *Garcinia* species that are much appreciated for their medicinal

and timber purposes include *G. atroviridis* Griff., *G. speciosa* Wall., *G. cowa* Roxb. and *G. dulcis* Kurz (Lim *et al.*, 1986). *G. atroviridis* contains hydroxycitric acid (HCA), especially in the fruits and leaves, which regulates enzymes involved in the hydrolytic pathway for conversion of fat or fatty acid into energy (Te-chato, 1997).

There have been very few reports of *in vitro* studies of other species within the *Clusiaceae*. Te-chato (1997) reported culture conditions for callus induction and multiple shoot formation from seeds, young leaves and shoot tip cultures derived from *in vitro*-germinated apomictic seedlings of *G. speciosa* and *G. atroviridis*. Sujaree and Te-chato (1996) described micropropagation of *G. atroviridis* from cultured shoot tips of *in vitro*-germinated apomictic seedlings. Induction of roots from individual shoots from these cultures was described by Suraninphong and Te-chato (1998).

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7.1 *Garcinia mangostana* Mangosteen

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1. Introduction

1.1. Botany and history

Mangosteen is a dioecious evergreen tree, 6–25 m, with a straight trunk, up to 25–35 cm in diameter, and with symmetrically arranged branches, which form a regular, pyramidal crown (Yaacob and Tindall, 1995). The bark is dark brown or nearly black, with a tendency to flake. The fruits are formed parthenogenically, i.e. without fertilization (Lim, 1984). The species is dioecious; however, only female trees are known. The female flowers are 5–6 cm in diameter with four sepals, four petals, several tiny rudimentary stamens and lobed stigma (Te-chato *et al.*, 2000). The sepals are large and persistent on the head of fruits through ripening, while the four petals shrivel and drop just after full blooming (Yaacob and Tindall, 1995). The flowering season of the tree varies according to location. From flowering to ripening usually requires 4–6 months. The fruit is a globular, indehiscent berry and either spherical or slightly flattened (Yaacob and Tindall, 1995). The diameter of the fruit ranges from 3.5 to 8.0 cm and weight varies from 75 to 150 g, depending on the age of the tree and its geographical location. The fruit apex is crowned with five to eight flat lobes of the stigma, which are woody, and the

rind, approx. 1 cm thick, is deep red and fibrous, and stains hands and clothes. The edible pulp or aril consists of four to eight white or ivory-coloured segments, some of which contain developed or undeveloped apomictic seeds. The embryos develop from the inner integument and there can be one or more in each seed (Lim, 1984).

The mangosteen was first domesticated in Thailand or Myanmar at least 2000 years ago (Corner, 1952), and later spread to other tropical areas. Trees grow in a range of soil types and locations. Distribution of mangosteen followed the spread of human settlements during the early period of population expansion in South-east Asia, but was limited by the recalcitrance of the seeds.

1.2. Importance

The mangosteen is an important export crop to many international markets. Fresh fruits command a high price in Hong Kong, Japan and the European Union (EU). Annual production has not been recorded by the Food and Agriculture Organization (FAO) and the mangosteen is considered to be a minor fruit crop wherever it is grown. In Malaysia, production has declined annually since the late 19th century; however, there has been a small increase in production of mangosteen

in Thailand (Yaacob and Tindall, 1995). Commercial production of mangosteen is well developed in the eastern and southern provinces of Thailand. A few medium- to large-scale growers export mangosteen to the EU and Japan.

1.3. Breeding and genetics

There are no reports of improvement of mangosteen by conventional breeding. There appear to be no distinct mangosteen cultivars. The different morphological characteristics of the fruit in different regions might be due to climatic and soil factors. The seeds of mangosteen and many other *Garcinia* species are formed apomictically (Corner, 1952), and thus all the plants are homogeneous and consist of a single genotype (Horn, 1940). The chromosome number of the mangosteen has been reported to be $2n = 4x = 56-76$, 88-90, 96 and 120-130 (Verheij, 1992). Jong (1973) and Richards (1990), quoted by Yaacob and Tindall (1995), indicated that the mangosteen is a polyploid and possibly the allotetraploid derivative of *G. malaccensis* T. Anderson ($2n = ?42$) and *G. hombroniana* Pierre ($2n = ?48$). There are several obvious breeding constraints: (i) low seed production; (ii) long gestation period; (iii) gamboge; (iv) sensitivity to drought; (v) poor root system; (vi) slow growth; and (vii) apomixis.

1.3.1. Major breeding objectives

Drought tolerance. Within the genus, mangosteen is most sensitive to drought for 3 to 5 years after germination. Shade trees or artificial shade must be provided during early growth.

Tree architecture. Mangosteen trees raised from seed normally have a high canopy and require from 7 to 12 years to fruiting. At this stage the tree has a straight trunk, 4-6 m long and 10-15 cm diam. The branches are arranged symmetrically to form a regular, pyramid-shaped crown.

Fruit quality. There are two problems concerning mangosteen fruit quality: (i) gam-

boge, the symptoms of which include hardening of the fruit pulp, which later turns reddish brown, while the pericarp and aril are both discoloured by a yellow resin; the aril also becomes bitter in taste; and (ii) fruit cracking, which may occur during wet weather due to an excessive uptake of water over a short period.

Rootstock. There is no documented information on effects of rootstocks on production and on stock-scion relationships. Generally, all *Garcinia* species except mangosteen can adapt well to drought and semi-arid zones since they have good root systems, which develop both horizontally (soil surface) and vertically (deep beneath the surface). At their early growth stage, they require no shade and frequent watering is unnecessary. Because of these characteristics, these species have been used as rootstocks of mangosteen for dry areas.

1.3.2. Breeding accomplishments

In the absence of a pollen source and because of the apomictic nature of mangosteen seeds, breeding is impossible. Genetic erosion of mangosteen has probably been severe throughout South-east Asia, and there are no truly wild populations. Other *Garcinia* species, e.g. somkhag (*G. atroviridis*), pawa (*G. speciosa*), ma-pood (*G. dulcis*), cha-muang (*G. cowa*) and ma-dun (*G. schombbergkiana* Pierre), are grown as 'dooryard' trees. Morphological characteristics of several species were described by Te-chato *et al.* (2000) (Table 7.1.1).

2. Molecular Genetics

2.1. Molecular markers

There have been a few reports involving the use of protein and DNA markers for mutagenesis studies, assessment of genetic diversity and determining somaclonal variation. Peroxidase and esterase have been used as markers for determining mutation of tissue culture-derived plants following mutagen treatments (Te-chato and Phrommee, 1999a;

Table 7.1.1. Morphological characteristics of some economically important *Garcinia* spp. in Thailand (from Te-chato *et al.*, 2000).

Characteristics	<i>G. cowa</i> Cha-muang	<i>G. speciosa</i> Pawa	<i>G. atroviridis</i> Somkhag	<i>G. mangostana</i> Mangosteen	<i>G. dulcis</i> Ma-phut	<i>G. schomburgkiana</i> Ma-dun
Flowering	Jul.–Sep.	Jul.–Sep. Jan.–Mar.	Jul.–Sep.	Feb.–Apr.	Jul.–Sep.	Jan.–Mar. (–Jun.), Jul.–Sep.
Latex or resin	Yellow	White	Yellow	Yellow	White	Yellow
Petal colour	Light yellow	Cream–light yellow	Purple	Red	Light green	Pink
Stigma	–	Stipitate	Sessile	Sessile	Stipitate	–
Stigma surface	–	Smooth	Corrugated	Corrugated	Corrugated	–
Stigma lobe	–	20% diameter	80% diameter	20% diameter	20% diameter	–
Stamen mass	Clustered	Lobed	Round	Isolated	Lobed	Clustered
Female flower	–	No staminodes	Staminodes	Staminodes	Staminodes	–
Pollen viability	96–100%	93–100%	3–5%	0.1–1%	0%	95–100%
Fruit shape	–	Oval	Globose	Globose	Globose	–
Fruit colour	–	Red	Green	Purple	Yellow	–
Fruit surface	–	Smooth	Wrinkled	Smooth	Lobed	–
Fruit flavour	–	Astringent	Sour	Sweet/sour	Sour	–

Te-chato and Sujaree, 1999). Polymerase chain reaction (PCR)-based random amplified polymorphic DNAs (RAPDs) have also been used for the same purposes (Te-chato, 1998a).

Nazre *et al.* (1998), using amplification of the *trnL-trnF* intergenic spacer region of chloroplast DNA, analysed the phylogeny of *Garcinia* species in the Pasoh forest of Malaysia. Diversity of *Garcinia* spp. and interspecies relationships were studied using amplified chloroplast DNA, and the results indicated that mangosteen is closely related to pawa, followed by somkhag, maphut and ma-dun (Te-chato *et al.*, 2000). Sando *et al.* (2001) used RAPDs to assess genetic diversity in mangosteen in northern Queensland. RAPD markers have also been utilized to determine clonal fidelity of callus and regenerants from young leaf cultures (Te-chato, 2000).

3. Micropropagation

Conventional vegetative propagation of mangosteen is difficult, as cuttings cannot be rooted and grafted buds often do not take, due to fermentation of the yellow resinous latex that is released when the cortex is injured (Almeyda and Martin, 1976).

Early micropropagation studies involved stimulation of multiple shoots from *in vitro*-germinated (apomictic) seeds. Micropropagation of mangosteen has been reported from seedling shoot tips (Goh *et al.*, 1988; Te-chato and Aengyong, 1988; Normah *et al.*, 1995). To enhance proliferation of shoot buds, a two-phase culture medium has been very successful. The lower semi-solid phase consists of woody plant medium (WPM) (Lloyd and McCown, 1981) with 1.39 mM polyvinylpyrrolidone (PVP) and 0.45 μ M benzyladenine (BA). Clusters of shoot buds are transferred on to the lower semi-solid medium. After 1–2 weeks, 5–10 ml of liquid half-strength Murashige and Skoog (1962) (MS) medium containing 0.32 μ M naphthaleneacetic acid (NAA) and 0.13 μ M BA is added to the cultures (Rakphurk and Te-chato, 1998). Axillary buds proliferate on this medium and shoots elongate rapidly.

Elongated shoots can be rooted efficiently (100%) by dipping the base of each excised shoot in 4.4 mM filter-sterilized indolebutyric acid (IBA) in the dark for 15 min (Te-chato and Sujaree, 1997). Root induction medium consists of WPM with 0.05–0.25% activated charcoal, 1.11 μ M BA and 1.39 mM fluorglucinol. Shoots are maintained in darkness for 2 weeks for root primordia induction. Root elongation occurs after transfer to a 16 h photoperiod (25 μ mol/m²/s provided by fluorescent lights).

4. Somatic Cell Genetics

4.1. Regeneration

4.1.1. Somatic embryogenesis

Somatic embryogenesis has been reported from cultured young purple leaves on semi-solid MS medium supplemented with 2.2 μ M BA, 2.3 μ M thidiazuron (TDZ) and PVP (Te-chato *et al.*, 1995a). Van Minh *et al.* (2001) reported somatic embryogenesis from seed pieces on plant growth medium supplemented with coconut water (CW). Details of somatic embryo development are not available.

4.1.2. Organogenesis

Induction

Direct shoot formation. Organogenesis has been reported from seeds and leaves of field-grown and *in vitro* plants. Adventitious shoot induction occurs from seed pieces on semi-solid MS medium supplemented with 40 μ M BA and 2.5 μ M NAA (Normah *et al.*, 1995) or on medium with 25 μ M BA for intact seeds. After culture on medium containing 25 μ M BA for 3–4 weeks, adventitious shoots can be subcultured on to medium containing 4.5 μ M BA.

Juvenile or red leaves from either field-grown mature trees or proliferating shoot cultures (2–4 cm) have also been used as explants. Direct shoot bud induction depends on culture medium, phytohormones, size and orientation of leaf explant (Goh *et al.*, 1990), leaf explant source, exci-

sion treatments of the explants (Goh *et al.*, 1994) and ethylene content in the culture vessel (Goh *et al.*, 1997; Lakshmanan *et al.*, 1997). High-frequency direct shoot bud induction has been obtained from 3 mm transverse sections of 10-day-old leaves on semi-solid WPM with 20 μ M BA and 20 g/l sucrose.

Adventitious shoot buds have also been induced from *in vitro* leaves in half-strength MS liquid medium supplemented with 0.13 μ M BA and 0.32 μ M NAA (Fig. 7.1.1). Adventitious shoot meristems develop directly from the epidermis and from vascular tissue (Te-chato *et al.*, 1992). Wounding of the midrib of *ex vitro* young red leaves, without complete severance, triggers shoot bud induction on leaves cultured on WPM supplemented with 20 μ M BA. Direct shoot bud regeneration from field-grown seedlings occurred more frequently than from *in vitro* shoots (Goh *et al.*, 1994).

Organogenic callus. Caulogenic callus has been induced from seeds or leaves forming clusters of shoots in two-phase medium on semi-solid induction medium consisting of MS medium supplemented with 3% sucrose, 1.39 mM PVP, 2.2 μ M BA and 2.3 μ M TDZ (Te-chato *et al.*, 1995a,b; Te-chato and Lim,

1999, 2000). The upper liquid phase consists of half-strength MS medium with 0.13 μ M TDZ alone or with 0.13 μ M BA + 0.32 μ M NAA (Te-chato *et al.*, 1995b). Caulogenic callus is nodular.

Maintenance. Organogenic callus proliferation can occur on induction medium or on medium with two times the concentration of organic composition. Culture bottles (55 mm diameter, 110 mm height) favour callus proliferation, while callus in Petri dishes produces leaf primordia due to the rich CO₂ concentration (Sawadipanee *et al.*, 1997). NAA and 2,4-dichlorophenoxyacetic acid (2,4-D) promote tissue necrosis and production of phenolic compounds (Te-chato *et al.*, 1995a).

Shoot development. Following transfer of callus on to shoot initiation medium consisting of WPM supplemented with 3% sucrose, 1.39 mM PVP and 0.45 μ M BA in Petri dishes, shoot primordia develop after 1 month.

4.1.3. Protoplast isolation and culture

Te-chato (1997) and Moosikapala and Te-chato (2000) described the isolation and culture of protoplasts from young leaves of



Fig. 7.1.1. Adventitious shoot buds from *in vitro* leaves in $\frac{1}{2}$ MS liquid medium supplemented with 0.13 μ M BA and 0.32 μ M NAA.

somkhag (*G. atroviridis*). Young leaves of mangosteen have also been used as a source of protoplasts. *In vitro* leaves (purple or red for mangosteen and green for somkhag) were used after pre-treating for 24 h in the dark. Leaf strips were incubated with 10 ml enzyme solution (1.0% (w/v) Pectolyase Y-23, 4% (w/v) cellulase Onozuka RS, 2% (w/v) macerozyme, 13% (w/v) mannitol and MS salts). Following 3–4 h incubation at 50 rpm under diffuse illumination at 28°C, the incubation mixture was passed through sterile nylon filtration fabric (60 µm) and the mixture was centrifuged at 800 rpm for 3 min. The protoplasts were washed once with MS salts supplemented with 13% (w/v) mannitol, layered in 21% (w/v) sucrose solution, washed once with medium and resuspended in 1 ml of medium. Protoplast yield was $1\text{--}5 \times 10^6$ /g fresh weight (FW). Leaf protoplasts are either anthocyanin-containing or chlorophyll-containing. Liquid MS medium supplemented with 2.22 µM BA, 2.25 µM TDZ, 13% (w/v) mannitol and 3% sucrose promoted protoplast division. After 4 days of culture the first protoplast divisions were observed at a frequency of 1–2%; however, sustained cell division has not been obtained.

4.2. Genetic manipulation

4.2.1. Mutation induction and somaclonal variation

Somaclonal variation has been observed following *in vitro* culture of seed on modified MS medium supplemented with 25 µM BA (Te-chato, 1998b; Fig. 7.1.2): chimeras (6×10^{-4}), malformed or closed funnel leaves (6×10^{-3}) and chlorosis (3×10^{-2}). Only the chimeras were genetically stable. To increase the frequency of mutation induction in mangosteen, Te-chato (1998b) and Te-chato and Phrommee (1999b) reported irradiation- and ethyl methanesulphonate (EMS)-induced mutation of young *in vitro* leaves. The optimum dosage for gamma irradiation (^{60}Co) is 10 Gy, for ultraviolet light it is 20,000 J/m²/s and for X-ray irradiation it is 20 Gy. Meristematic nodular callus was treated with various concentrations of EMS, and 0.5% EMS appeared to be optimum (Te-chato and Phrommee, 1999a). RAPD analyses of calluses from treated leaves showed polymorphisms, which were correlated with inhibition of callus formation (Te-chato, 1998a). Morphological changes of the first regenerated irradiated plantlets included



Fig. 7.1.2. Somaclonal variants obtained from apomictic seeds in medium supplemented with 25 µM BA.

short stems, emarginate leaf apex, serrate leaf margin, whorled phyllotaxy and lateral branching (Phrommee and Te-chato, 1998; Fig. 7.1.3).

Te-chato and Sujaree (1999, 2000) reported the effect of colchicine on induction of polyploidy and mutation of adventitious shoot buds and callus. The number of chromosomes following colchicine treatment could not be determined due to their small size; however, 0.075–0.1% colchicine caused an increase in size and colour of guard cells. Peroxidase and esterase markers indicated that there were differences between treated and non-treated plantlets. Morphological abnormalities of plantlets induced from treated callus included the production of new shoots on intact leaves, a large number of roots and three leaves per whorl.

4.2.2. Somatic hybridization

In order to transfer drought tolerance from somkhag, protoplast fusion between this species and mangosteen was attempted.

Protoplasts of mangosteen at a density of $2 \times 10^5/\text{ml}$ were mixed with protoplasts of somkhag at a density of $1 \times 10^5/\text{ml}$. Division of somkhag protoplasts was observed; however, division of mangosteen and hybrid protoplasts did not occur.

4.2.3. Genetic transformation

Organogenic callus and young red leaves were co-cultured with a suspension of *Agrobacterium tumefaciens* strain LBA4404 for 10–15 min followed by co-culture on medium without antibiotic for 48 h. Two different gene constructs were used: (i) pBI121, which contains the selectable marker neomycin phosphotransferase II (nptII) and the scorable marker glucuronidase (gus); and (ii) pTok233, which contains the two selectable markers nptII and hygromycin phosphotransferase (hpt). The nptII and gus genes in pBI121 are driven by the 35S promoter. The nptII gene in pTok233 is driven by the nos promoter while the hpt gene is driven by the 35S promoter. *A. tumefaciens* was eliminated with



Fig. 7.1.3 Morphological changes of the first regenerated irradiated plantlets: (A) phyllotaxy change; (B) emarginate leaf apex; (C) serrate leaf margin; (D) three leaves per whorl; (E) branching.

200 mg/l cefotaxime. Leaves and callus were transferred on to callus and shoot induction media, respectively. Media were supplemented with 50–100 mg/l kanamycin to select for pBI121 and with 50 mg/l hygromycin to select for pTok233. Transformation with pBI121 resulted in a higher frequency of calluses resistant to kanamycin; regeneration has not been observed.

5. Conclusions

Mangosteen, because it is parthenocarpic like *Musa*, should benefit from the application of biotechnology for improving this crop. Efficient protocols for *in vitro* regeneration of mangosteen from apomictic seeds and leaves are available, and preliminary studies have been undertaken to genetically modify this crop species. Somaclonal variants and off-types from mutagenesis studies have been identified. Somatic hybridization

and genetic transformation have been attempted; however, the results have been disappointing, probably due to the relatively low efficiency of the organogenic pathway. Optimizing somatic embryogenesis with mangosteen is essential in order to apply cell manipulation techniques to this crop. Molecular markers for mangosteen and related *Garcinia* spp. could be very useful for determining the ancestry of mangosteen. It might then be possible to re-create mangosteen by hybridizing the putative parents.

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8

Ericaceae

The *Ericaceae* family consists of small trees or shrubs and a few lianas in approx. 100 genera and 1350 species (Watson and Dallwitz, 1992 onwards). Species range from the subpolar regions to the subtropics and highland tropics. Edible berries are produced by several species

in addition to cranberry and blueberry, e.g. *Arctostaphylos* spp., *Gaylussacia* spp. and within *Vaccinium*: bearberry, bilberry, buckleberry, whortleberry, etc. A few species are important as ornamentals, e.g. *Erica* spp., *Rhododendron* spp., *Arbutus* spp. and *Pieris* spp.

Reference

Watson, L. and Dallwitz, M.J. (1992 onwards) The families of flowering plants: descriptions, illustrations, identification and information retrieval. Version 14 December 2000. <http://biodiversity.uno.edu/delta/>

8.1 *Vaccinium* spp. Blueberry

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1. Introduction

1.1. Botany and history

All cultivated blueberries belong in the section *Cyanococcus* of the genus *Vaccinium* of the heath family *Ericaceae* (Galletta and Ballington, 1996). Species within this section are often called the 'true' or cluster-fruited blueberries (Camp, 1945). Wild representatives of *Cyanococcus* are found solely in North America (Hancock and Draper, 1989). The most recent classification of the *Cyanococcus* species, described by Galletta and Ballington (1996) and based on Vander Kloet (1988) with some additional revisions (Bruederle and Vorsa, 1994), includes a total of seven diploid species (*V. boreale* Hall & Aald., *V. corymbosum* L., *V. darrowi* Camp, *V. elliotii* Chapm., *V. myrtilloides* Michx., *V. pallidum* Ait. and *V. tenellum* Ait.), six tetraploid species (*V. angustifolium* Ait., *V. corymbosum*, *V. hirsutum* Buckley, *V. myrsinites* Lam., *V. pallidum* and *V. simulatum* Small) and two hexaploid species (*V. ashei* Reade and *V. constablaei* Gray), with *V. corymbosum* and *V. pallidum* occurring at diploid and tetraploid levels.

Blueberry species are also commonly grouped according to stature and referred to as the lowbush, highbush and rabbiteye types. Lowbush plants are rhizomatous with stems from 0.30 to 0.60 m; highbush plants

are crown forming and generally maintained between 1.8 and 2.5 m; rabbiteye plants are crown forming, but are also notable for suckering to varying degrees, and are maintained between 2.0 and 4.0 m (Hancock and Draper, 1989; Galletta and Ballington, 1996).

Of the major fruit crops, blueberry has been domesticated most recently, having been accomplished entirely within the 20th century. Wild lowbush blueberry plants were probably the first blueberries to be cultivated, with native North Americans burning the heaths to increase yield (Eck and Childers, 1966; Hancock and Draper, 1989). Commercial lowbush blueberry production, mainly *V. angustifolium* but also including *V. myrtilloides*, began in the 1860s in New England with the canning of fruit (Ballington, 2001). Domestication of the highbush blueberry (mainly *V. corymbosum*) began in New Jersey in the early 1900s through the efforts of US Department of Agriculture (USDA) botanist F.V. Coville and blueberry enthusiast E.C. White (Eck and Childers, 1966; Hancock and Draper, 1989; Galletta and Ballington, 1996; Ballington, 2001). The first commercial fruit from hybrid highbush plants was harvested in 1916 (Coville, 1921; Hokanson, 2001). Efforts to domesticate rabbiteye blueberry began around 1893 with the transplantation of native seedlings by M.A. Sapp to his farm in

north-western Florida (Hancock and Draper, 1989; Galletta and Ballington, 1996; Ballington, 2001).

Currently, commercially grown blueberries can be divided into five major groups of three different ploidy levels: (i) the lowbush types (2x and 4x), which include managed wild populations of *V. angustifolium*, *V. myrtilloides* and *V. boreale* and improved lowbush cultivars; (ii) the highbush types (4x), which include *V. corymbosum* wild selections and hybrid cultivars, often with small percentages of *V. angustifolium* in their parentage; (iii) the southern highbush types (4x), which are predominantly highbush *V. corymbosum* germplasm but which have the low-chilling species *V. darrowi* in their parentage, as well as *V. angustifolium* and in some cases *V. ashei* and *V. tenellum* (Lyrene, 1990; Ballington *et al.*, 1991); (iv) the half-high types (4x), which are species hybrids or back-cross derivatives of lowbush–highbush hybrids, usually involving *V. angustifolium* and *V. corymbosum* parentage; and (v) the rabbiteye types (6x), which are all wild selections and hybrid cultivars of *V. ashei* (Galletta and Ballington, 1996; Hokanson, 2001).

1.2. Importance

Blueberry is a high-value crop which can thrive on acidic, imperfectly drained sandy soils, once considered worthless for agricultural crop production. North America is the major producer of blueberries. According to Ballington (2001) and statistics from the Food and Agriculture Organization (FAOSTAT, 2004), current North American total annual production of commercial blueberries approaches 200,988 t, with approximately 60% of that being cultivated (standard highbush, southern highbush, half-high and rabbiteye) blueberries and about 40% being the managed wild, or semicultivated, lowbush blueberries. The total area devoted to growing commercial blueberries in North America is approx. 74,000 ha, consisting of 53,000 ha of managed wild stands of lowbush blueberries and 21,000 ha of cultivated blueberries (Ballington, 2001). Most of the US blueberry crop is grown in 12 states: Alabama,

Arkansas, Florida, Georgia, Indiana, Maine, Michigan, New Jersey, New York, North Carolina, Oregon and Washington; Michigan, Maine, New Jersey and Oregon are the top producers (Hancock and Draper, 1989; National Agricultural Statistics Service, 2000). The Canadian blueberry crop is grown mainly in British Columbia, Nova Scotia, New Brunswick and Quebec (Hancock and Draper, 1989). Worldwide, in addition to North America, commercial industries now exist in Europe, Australia, New Zealand, Chile and Argentina (Munoz, 1993; Galletta and Ballington, 1996; Ballington, 2001).

Biomedical and epidemiological research suggests that the dietary antioxidants contained in fruits and vegetables may play an important role in preventing disease (Wang *et al.*, 1996). Plant-derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, free radical scavengers, peroxide decomposers, enzyme inhibitors and synergists (Larson, 1988). Blueberries are one of the richest sources of antioxidants of all fresh fruits and vegetables (Prior *et al.*, 1998). Compared with other fruits, canned blueberries are also a good source of iron, fair source of vitamin A and about average in terms of protein, fat, carbohydrate, calories and calcium content (Anon., 1956; Galletta and Ballington, 1996). In addition, fresh blueberries are a fair source of vitamin C (Matzner, 1967; Galletta and Ballington, 1996).

1.3. Breeding and genetics

The principles, techniques and objectives of blueberry breeding have been reviewed by Darrow (1960), Moore (1965, 1966), Darrow and Scott (1966), Draper and Scott (1967), Galletta (1975), Lyrene (1988a), Ballington (1990), Luby *et al.* (1991), Galletta and Ballington (1996) and Ballington (2001). Because of a lack of fundamental sterility barriers between blueberry species of the same ploidy level (homoploid species), interspecific hybridizations have played, and continue to play, an important part in blueberry breeding programmes. Hybridizations between species of different ploidy levels (heteroploid crosses), although much more

difficult to achieve, have also been utilized in blueberry breeding (Galletta and Ballington, 1996; Ballington, 2001).

1.3.1. Major breeding objectives

The initial blueberry breeding objectives were mainly focused on the fresh fruit characters of sweet and excellent flavour, large size, dry scar and plumpness at maturity, light blue colour or bloom and vegetative growth adequate to support a large crop of berries (Coville, 1921; Galletta and Ballington, 1996). With the expansion of the blueberry industry in North America and worldwide, modern blueberry breeding objectives now include generating plants with broader soil and climatic adaptation, disease and pest resistance, easy mechanical harvesting, extreme early and late harvesting and good fruit quality, including longer storage life (Galletta and Ballington, 1996). Concerning broader soil adaptation, there is a need to breed blueberries for less acidic, well-drained upland mineral soils. Broader climatic adaptation refers, on the one hand, to adaptation to warm, long-growing season areas through lower chilling requirements and increased heat and drought tolerance and, on the other hand, to adaptation to colder production areas through increased bud and wood cold tolerance and delayed flowering to avoid early spring frosts, combined with earlier fruit maturation (Galletta and Ballington, 1996; Hokanson, 2001). Disease resistance refers to problems such as mummy berry, blueberry stunt, blueberry shoestring, leaf mottle, scorch and red ringspot viruses, stem blight, stem canker, botrytis, anthracnose, phomopsis twig blight, canker, alternaria and fruit rot, fusioccum canker and red leaf rose bloom (Galletta and Ballington, 1996). The major pests include the blueberry maggot, sharp-nosed leafhopper, blueberry aphid, oriental beetle, cranberry fruit worm, cherry fruit worm, plum curculio, blueberry bud mite and *Xiphonema* nematode (Galletta and Ballington, 1996). With regard to mechanical harvesting, the goal is to develop productive firm- and small-fruited cultivars (Ballington, 2001). Extreme early and late harvesting is of

interest because of the possibility of extending the marketing period when berry prices are at their peak or marketing when prices are rebounding (Ehlenfeldt, 1997). Good fruit quality refers to a combination of several fruit characters including large size, light blue colour (having a heavy coating of waxy bloom), a small, shallow, dry scar (opening in the fruit epidermal wall remaining after the berry is removed from the pedicel and principal site of entry for spores of postharvest decay organisms), firmness, good flavour and longer storage life (Galletta and Ballington, 1996).

1.3.2. Breeding accomplishments

Major increases in fruit size, fruit quality, productivity, disease resistance and bush adaptation have been achieved, utilizing a narrow genetic base and only a few generations of hybridization and selection (Galletta and Ballington, 1996). In the last 35 years, significant advances have also been made to broaden the germplasm base in highbush blueberries (Ballington, 2001). Wild $\times$ cultivated highbush crosses have been and continue to be used in breeding programmes in Michigan and North Carolina to develop cultivars suitable for mechanical harvesting (Ballington, 2001). Wild *V. corymbosum* selections have been used for many years for developing blueberry cultivars resistant to stem canker for the south-eastern USA (Ballington, 2001). *V. darrowi* and *V. darrowi* $\times$ *V. ashei* hybrids have been used to introduce genes for adaptation to warm growing areas (including genes for low chilling requirement and heat tolerance) into the standard *V. corymbosum* highbush background and have provided genes for high fruit quality (Hancock *et al.*, 1995; Lyrene, 1998; Ballington, 2001). This work has resulted in the release of about 20 cultivars of 'southern highbush'. The cold hardy 'half-high' cultivars from the Minnesota breeding programme have resulted from crosses involving *V. angustifolium* and *V. corymbosum*, and it has been estimated that about half the current standard highbush cultivars have some *V. angustifolium* germplasm in their pedigrees (Galletta and Ballington, 1996;

Ballington, 2001). With regard to rabbiteye blueberries, crosses between *V. ashei* and *V. constablaei* have resulted in the release of two hybrid cultivars (Ballington, 2001). Hexaploid southern highbush genotypes have been developed (Dweikat and Lyrene, 1989; Ehlenfeldt and Vorsa, 1993) and utilization of this germplasm appears to be a promising means for the future for combining the fruit quality and early ripening traits of highbush with the productivity and adaptation traits of rabbiteye (Ballington, 2001).

2. Molecular Genetics

2.1. Gene cloning

Molecular genetic research in blueberry began only recently. Because lack of cold hardiness and susceptibility to spring frosts have been identified as two of the most important genetic limitations of current blueberry cultivars (Moore, 1993), researchers have focused on identifying cold-responsive proteins from blueberry floral buds in the hope of identifying proteins that play a role in determination of cold hardiness. To date, all efforts to clone genes using traditional molecular genetic approaches from blueberry have followed from the identification and characterization of cold-responsive proteins known as dehydrins. Table 8.1.1 summarizes information on the dehydrin complementary DNA (cDNA) and genomic clones that have been isolated and characterized from blueberry to date.

Initially, Muthalif and Rowland (1994a) examined changes in protein levels associated with low temperature exposure in floral buds of two blueberry cultivars with different levels of cold hardiness. The cultivars used were relatively cold-tolerant *V. corymbosum* 'Bluecrop' and more cold-sensitive *V. ashei* 'Tifblue'. From profiles of total and soluble proteins, the levels of three proteins of 65, 60 and 14 kDa were observed to increase with low temperature exposure in floral buds of both cultivars during the winter such that they become the predominant proteins, visible on one-dimensional sodium dodecyl sulphate (SDS)-polyacrylamide gels (Muthalif and Rowland, 1994a) (Fig. 8.1.1).

Further characterization of the cold-responsive proteins revealed them to be members of a family of proteins known as dehydrins (Muthalif and Rowland, 1994a). Dehydrins are a group of heat-stable, glycine-rich plant proteins that are induced by environmental stimuli that have a dehydrative component, including drought, low temperature, salinity and seed maturation (Close, 1996). Also indicative of dehydrins is the presence of a highly conserved lysine-rich amino acid sequence (consensus sequence EKKGIMDKIKEKLPG) referred to as the K segment, which is often repeated several times (Close, 1996). The K segments are predicted to form amphipathic α -helices (Close, 1997), and amphipathic α -helices may have a role in stabilizing cell membranes against

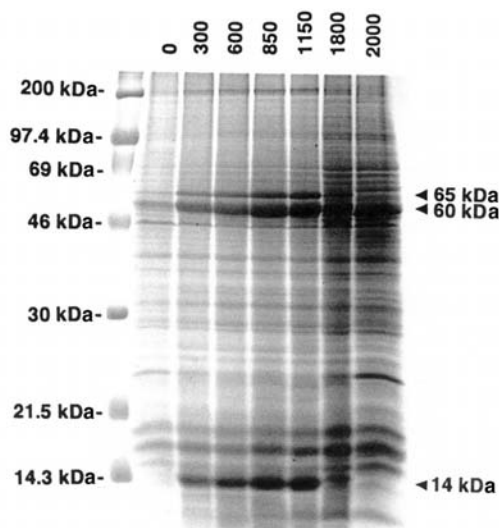


Fig. 8.1.1. Profiles of soluble proteins extracted from floral buds of *V. corymbosum* 'Bluecrop' (chilling requirement of approx. 1200 chill units) collected at various times during chill unit accumulation until the resumption of growth. Proteins were extracted and equal amounts of proteins were loaded from each time point and separated on a 12.5% gel by SDS-polyacrylamide gel electrophoresis (Muthalif and Rowland, 1994a). Chill units are indicated above each lane. In the far left lane are molecular weight markers. Arrows to the right mark the 65, 60, and 14 kDa polypeptides that accumulate with low temperature exposure and decrease with the resumption of growth. In this case, buds had begun to swell by about 1800 chill units.

Table 8.1.1. Summary of dehydrin cDNA and genomic clones isolated and characterized to date from blueberry.

Type of clone (size)	Source	Gene designation	Gene product	Genbank accession no.	Reference
cDNA (2.0 kb – full length)	RNA from floral buds of cold-acclimatized 'Bluecrop'	<i>bbdhn1</i>	60 kDa dehydrin	AF030180	Levi <i>et al.</i> , 1999
cDNA (0.6 kb – partial length)	RNA from floral buds of cold-acclimatized 'Bluecrop'	<i>bbdhn2</i>	Dehydrin	AF222738	Rowland <i>et al.</i> , 2004
cDNA (0.8 kb – partial length)	RNA from floral buds of cold-acclimatized 'Bluecrop'	<i>bbdhn3</i>	Dehydrin	AF222739	Rowland <i>et al.</i> , 2004
cDNA (0.9 kb – partial length)	RNA from floral buds of cold-acclimatized 'Bluecrop'	<i>bbdhn4</i>	Dehydrin	AF222740	Rowland <i>et al.</i> , 2004
cDNA (1.2 kb – partial length)	RNA from floral buds of cold-acclimatized 'Bluecrop'	<i>bbdhn5</i>	Dehydrin	AF222741	Rowland <i>et al.</i> , 2004
Genomic (174 bp)	PCR product from 'Bluecrop'	Unknown	Dehydrin-related		Levi <i>et al.</i> , 1999
Genomic (2.0 kb)	PCR product from selection Fl44B	Unknown	Dehydrin-related		Mehra <i>et al.</i> , 2001
Genomic (2.0 kb)	PCR product from selection W85-20	Unknown	Dehydrin-related		Mehra <i>et al.</i> , 2001
Genomic (1.8 kb)	PCR product from selection Fl44B	Unknown	Dehydrin-related		Mehra <i>et al.</i> , 2001
Genomic (1.3 kb)	PCR product from selection Fl44B	<i>bbdhn1</i>	60 kDa dehydrin		Mehra <i>et al.</i> , 2001
Genomic (1.3 kb)	PCR product from selection W85-20	<i>bbdhn1</i>	60 kDa dehydrin		Mehra <i>et al.</i> , 2001

freezing damage (Thomashow, 1999). The categorization of the cold-responsive proteins in blueberry as dehydrins was based on several factors, including their reaction to antiserum raised against the dehydrin-specific consensus peptide or K segment, their heat-stability and the similarity in amino acid composition of selected sequenced peptides from the cold-responsive proteins to dehydrins (Muthalif and Rowland, 1994a).

From densitometric scans of the protein gels along with bud cold hardiness evaluations, Muthalif and Rowland (1994a) concluded that, for the two cultivars tested, levels of the dehydrins appeared to correlate with cold hardiness levels. The largest increase in the dehydrin levels coincided with the largest increase in the level of cold hardiness and the most dramatic decline in cold hardiness occurred with the resumption of growth, as did the decline in levels of the dehydrins. Also, maximum level of cold hardiness and maximum level of the dehydrins were higher in 'Bluecrop' than in 'Tifblue'. This correlation in dehydrin and cold hardiness levels has held up in many subsequent studies (Muthalif and Rowland, 1994b; Arora *et al.*, 1997; Panta *et al.*, 2001) with a number of different cultivars and selections, including 'Berkeley', 'Gulfcoast', 'Climax' and *V. darrowii* selection Fla 4B, in addition to 'Bluecrop' and 'Tifblue'.

Peptide sequence information from the 65 and 60 kDa dehydrins of blueberry was used to synthesize degenerate primers for polymerase chain reaction (PCR) amplification of a part of a dehydrin gene (Levi *et al.*, 1999). One pair of primers, based on the amino acid sequences derived from the 65 kDa dehydrin, resulted in amplification of a 174 bp fragment. When this fragment was cloned and sequenced, the presence of a K box (EGGGLADKVKDKIHG) within the sequence confirmed that part of a dehydrin gene had been isolated.

The 174 bp PCR fragment was used to screen a cDNA library prepared from RNA from dormant, cold-hardy floral buds of 'Bluecrop' (Levi *et al.*, 1999). The buds for the cDNA library construction had been collected from field plants having acquired approxi-

mately 600 chill units, since previously it was shown that dehydrin levels are maximal by about 600–900 chill units (Muthalif and Rowland, 1994a). Hybridization with the 174 bp PCR fragment resulted in the isolation and purification of a clone with a 2.0 kb insert.

The 2.0 kb cDNA was sequenced and determined to be full length. Inspection of the sequence confirmed that it encodes a member of the dehydrin family of proteins. Like dehydrins (Close, 1996), the deduced protein is hydrophilic, has a preponderance of glycine residues, is rich in polar and charged amino acids (such as glutamine, aspartic acid, lysine, tyrosine, histidine, glutamic acid and arginine) and contains no phenylalanine or tryptophan. The deduced protein sequence contains five lysine-rich repeats or K boxes indicative of dehydrins. Because five high-confidence peptide sequences, ranging from 9 to 25 amino acids long, obtained from the 60 kDa dehydrin, exactly matched sequences encoded within the cDNA clone, it was concluded that the 2.0 kb dehydrin cDNA encodes the 60 kDa dehydrin (Levi *et al.*, 1999). The gene represented by this clone was named *bbdhn1* for blueberry dehydrin 1.

The 2.0 kb blueberry dehydrin cDNA has itself now been used as a probe to screen the cDNA library prepared from RNA from cold-hardened floral buds of 'Bluecrop' (Rowland *et al.*, 2004). In this screening, several positively hybridizing plaques were detected and, to date, four have been purified and completely sequenced. Analyses of the sequences confirm that they are all dehydrin cDNAs. All contain between two and five K boxes, all are hydrophilic and all are rich in glycine and polar and charged amino acids such as glutamine. All are similar but unique, since none of the sequences exactly match any of the other clones, including *bbdhn1*. None, except for *bbdhn1*, are full length as their open reading frames do not begin with an ATG start codon. The four partial-length cDNA clones have inserts that are 0.6, 0.8, 0.9 and 1.2 kb long and the genes represented by these sequences were named *bbdhn2*, *bbdhn3*, *bbdhn4* and *bbdhn5*, respectively. The cDNA sequences were entered

into the GenBank and assigned accession numbers AF222738–AF222741.

An alignment of the five dehydrin sequences revealed that they are very similar at the DNA and protein levels (Rowland *et al.*, 2004). The sequences are more conserved at the 3'/carboxy end than at the 5'/amino end. In fact, all five cDNA clones have the same 61-amino acid sequence at the carboxy end, suggesting that this sequence may serve an important function. As the sequences diverge toward the 5'/amino end, the dehydrins appear to differ from each other by a series of insertions/deletions and single base changes.

Most recently, the sequence of the 2.0 kb cDNA representing *bbdhn1*, which encodes the 60 kDa dehydrin from blueberry, was used to design primers to amplify and clone alleles of two dehydrin-related genes from the cold-sensitive and cold-tolerant parent plants, Fla 4B and W85–20, respectively, of a blueberry mapping population (Mehra *et al.*, 2001). One of the genes has alleles of 2.0 and 1.8 kb present in the mapping parents whereas the other gene has alleles of 1.3 kb in both mapping parents. The dehydrin-related gene with alleles of 2.0 and 1.8 kb in the parents is different from, but similar to, other previously characterized dehydrin clones. This gene was mapped to linkage group 12 of the current genetic linkage map of blueberry (Panta *et al.*, 2004). Sequences of the 1.3 kb alleles of the other dehydrin gene indicated that they encode the *bbdhn1* gene (L.J. Rowland, unpublished). The alleles from the cold-sensitive and cold-tolerant parent plants are very similar to each other, differing by only a few single nucleotide changes and one 5-base insertion/deletion near the 3' end of the gene. Primers based on the regions showing differences are currently being used to attempt amplifying polymorphic fragments for mapping purposes (L.J. Rowland, unpublished).

2.1.1. Marker-assisted selection

The development of genetic linkage maps and mapping quantitative trait loci (QTLs) in woody perennials have lagged behind that of herbaceous annual plants for a

number of reasons, including long generation times, high ploidy levels, lack of described Mendelian markers, self- and cross-incompatibility, inbreeding depression and recalcitrance to many molecular genetic and biochemical techniques exhibited by some woody perennials (Durham *et al.*, 1992; Rowland and Levi, 1994). This is unfortunate because marker-assisted selection should be particularly beneficial for blueberry for following complex phenotypes in breeding populations. A great deal of time, labour and land resources could be saved if potentially low-value genotypes could be eliminated at the seedling stage before field planting (Qu and Hancock, 1997). Despite the difficulties of working with blueberry, much progress has been made in the last few years in developing molecular markers for fingerprinting cultivars and selections, analysis of genetic relationships in breeding material and constructing genetic linkage maps.

2.1.2. Molecular markers

Before the development of molecular markers, genetic analysis in blueberry, as in many perennial, outcrossing plant species, was severely constrained by the limited number of simply inherited genetic markers. Only four simply inherited traits had been described (Lyrene, 1988b): glaucous leaf (dominant) versus non-glaucous in *V. angustifolium* (Aalders and Hall, 1963); blue or black berry (dominant) versus albino berry in *V. myrtilloides* (Aalders and Hall, 1962), *V. angustifolium* (Hall and Aalders, 1963) and *V. corymbosum* (Ballinger *et al.*, 1972); green seedling (dominant) versus lethal albino seedling in *V. corymbosum* (Draper and Scott, 1971); and normally pigmented red autumn foliage, reddish-brown bud scales and black ripe fruit (dominant) versus anthocyanin-deficient yellow autumn foliage, whitish-green bud scales and greenish-white ripe fruit in *V. elliottii* (Lyrene, 1988b). With the development of biochemical and DNA marker technologies, many new alternative methods have become available for generating large numbers of genetic markers.

Protein markers. Hill and Vander Kloet (1983), in their initial efforts to identify isozyme markers for genetic studies in blueberry, reported limited variation in four enzyme systems (esterase, malate dehydrogenase, peroxidase and phosphoglucose isomerase) among four *Vaccinium* sections, including *Cyanococcus*. The authors believed that their difficulties in isozyme analysis in blueberry were due to phenolic interference. After refinement of techniques to reduce or eliminate this phenolic interference, several researchers reported successful recovery of blueberry isozymes by starch gel electrophoresis. Vorsa *et al.* (1988) analysed leaf tissue extracts from diploid, tetraploid and hexaploid *Cyanococcus* species for isozyme polymorphisms in 12 enzyme systems, of which four (malate dehydrogenase, phosphoglucose isomerase, 6-phosphogluconate dehydrogenase and isocitrate dehydrogenase) yielded interpretable banding patterns and eight showed activity but unclear banding. In addition, single-gene Mendelian inheritance was indicated by allozyme segregation ratios in the progeny of controlled diploid crosses. Van Heemstra *et al.* (1991) studied the inheritance at the diploid level of nine of the isozyme loci governing the enzyme systems, aconitase, aldolase, alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, leucine aminopeptidase, malate dehydrogenase, 6-phosphogluconate dehydrogenase and phosphoglucomutase. Single-gene inheritance was indicated in these systems, as well. In addition, two pairs of linked loci, *Pgi-2/Lap-1* and *Pgm-2/6-Pgd-2*, were found, the first to be reported in blueberry; and they were independent from each other. Krebs and Hancock (1989) used isozyme markers to investigate the mode of inheritance in tetraploid *V. corymbosum* and reported that *V. corymbosum* has tetrasomic inheritance in the four enzyme systems analysed: malate dehydrogenase, phosphoglucose isomerase, 6-phosphogluconate dehydrogenase and aspartate aminotransferase. Bruederle *et al.* (1991) extended isozyme analyses of 20 loci to the investigation of population genetic structure among diploid blueberry species *V. elliotii*, *V. myrtilloides* and *V. tenellum*. They found that the

diploid species exhibit high levels of variation within populations as expected for highly self-sterile, outcrossing taxa. All populations were in Hardy-Weinberg equilibrium with slight heterozygote excess observed in the more broadly distributed *V. tenellum* and *V. myrtilloides*. Hokanson and Hancock (1998) examined levels of allozymic diversity in native Michigan populations of diploid *V. myrtilloides* and the tetraploids *V. angustifolium* and *V. corymbosum*. Six enzyme systems were evaluated and levels of heterozygosity and the number of alleles were averaged over seven polymorphic isozyme loci. The level of heterozygosity and number of alleles per locus were significantly lower in the diploid *V. myrtilloides* than in the tetraploids, as has been found in other studies comparing closely related diploid and tetraploid species.

DNA markers. Isozyme analysis is limited by the relatively small number of loci that can be examined. DNA markers, on the other hand, can provide large numbers of polymorphisms for genetic analyses. Many methods are currently available for generating DNA markers, each with its own advantages and disadvantages. To date, five types of DNA markers have been used for genetic analyses in blueberry: restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), expressed sequence tag (EST)-PCR and cleaved amplified polymorphic sequences (CAPS) derived from EST-PCR markers.

RFLPs were the first broadly applicable DNA marker to be developed (Botstein *et al.*, 1980). RFLPs represent heritable changes in fragment length of genomic DNA arising from digestion with specific restriction enzymes. Their detection generally depends upon Southern hybridization of the digested DNA with a radioactively labelled cloned DNA referred to as a probe. Haghghi and Hancock (1992) performed RFLP analyses on various genotypes representing the blueberry species *V. corymbosum*, *V. angustifolium*, *V. darrowi* and *V. ashei*, using chloroplast-specific and mitochondrial-specific gene probes. After digestion of *Vaccinium* DNA with sev-

eral different restriction enzymes, no polymorphisms were detected in the chloroplast genome, whereas high levels of polymorphism were observed in the mitochondrial genome.

Several PCR-based methods for detecting DNA polymorphisms have been developed, including RAPD analysis, which was developed simultaneously in two independent laboratories (Welsh and McClelland, 1990; Williams *et al.*, 1990). RAPD technology employs short 10-base primers of arbitrary nucleotide sequences (> 50% GC) for amplification of multiple fragments of genomic DNA that are easily visualized on ethidium bromide-stained agarose gels. RAPD analysis has several advantages over RFLP analysis. No prior knowledge of the genome of the organism being studied, such as sequence data or available cloned DNA, is required. The procedure is simple and does not require the use of radioactive probes, and the procedure requires much less DNA than RFLP analysis. Disadvantages, however, include difficulty in reproducing results between laboratories and the fact that most RAPD markers are dominant and thus less informative in some types of genetic analyses than co-dominant markers like isozyme and RFLP markers.

Aruna *et al.* (1993) and Levi *et al.* (1993) reported successful amplification of RAPD markers from blueberry DNA. Aruna *et al.* (1993) reported good results from DNA of native selections and improved cultivars of rabbiteye blueberry (*V. ashei*) using essentially the same amplification conditions described by Williams *et al.* (1990). Levi *et al.* (1993) described an RAPD protocol for the reproducible amplification of RAPD markers from several different woody plants including blueberry, cherry, peach, pear and apple. This procedure utilized a PCR buffer that contains levels of gelatin (0.1%) and a non-ionic detergent (1% Triton-X-100) higher than typically described, together with a higher annealing temperature of 48°C. Since this initial protocol was described, researchers have recommended replacing the gelatin in the buffer with 0.1% bovine serum albumin, after finding that the gelatin source could affect results (Stommel *et al.*, 1997).

Another type of PCR-based molecular marker utilizes a subclass of repetitive DNA sequences containing iterations of very short simple sequence repeats (1–5 bp) called SSRs or microsatellites (Weber and May, 1989). Microsatellites are highly abundant in plant genomes and their loci are polymorphic (Wang *et al.*, 1994). Once microsatellite loci are identified, cloned and sequenced, a pair of primers can be synthesized based on sequences flanking the microsatellites and used to amplify the co-dominant, simple sequence length polymorphisms (SSLPs). A variation on this technique has been described which, like RAPD, does not require prior knowledge of the analysed genome. Zietkiewicz *et al.* (1994) described this technique, which utilizes short repeats found to be abundant in a large number of organisms, such as the (CA)_n repeats, as priming sites for ISSR PCR. With this technique, primers are synthesized that are anchored at the 3' or 5' terminus of the repeat by extending into a hypothetical flanking sequence by 2–4 bases. For example, one such anchored primer is (CA)₈RG. Levi and Rowland (1997) used their RAPD protocol (Levi *et al.*, 1993), modified with an increase in the annealing temperature to 59°C, to test 100 such SSR-anchored primers for their usefulness at amplifying blueberry DNA. Fifteen of the 100 tested primers resulted in good amplification.

PCR markers derived from ESTs, termed EST-PCR markers, have a number of advantages for genetic studies. First, they target expressed genes; thus, they should be particularly useful for QTL mapping. If an EST marker is linked to a QTL, it is possible that the gene itself, from which the EST marker was derived, controls the trait in question. Secondly, because they are derived from gene coding regions, which are more likely to be conserved across populations and species than non-coding regions, EST markers should be useful for comparative mapping studies. Furthermore, EST-based markers have the potential for being co-dominantly inherited. However, generally, amplification using EST-specific primers must be followed by either digestion with restriction enzymes to generate CAPS mark-

ers, heteroduplex analysis or single-stranded conformational polymorphism (SSCP) analysis to detect polymorphisms. Rowland *et al.* (2003a) designed 30 PCR primer pairs from blueberry ESTs and tested them in amplification reactions with genomic DNA from 19 blueberry genotypes. Fifteen of the 30 primer pairs resulted in amplification of polymorphic fragments that were detectable directly after ethidium bromide staining of agarose gels. Several of the monomorphic amplification products were digested with the restriction enzyme *AluI* and approximately half resulted in polymorphic-sized fragments or CAPS markers. The EST-PCR primer pairs developed in this study plus some additional blueberry EST-PCR primer pairs were tested for their ability to amplify fragments in related *Ericaceae*, cranberry and rhododendron (Rowland *et al.*, 2003b). Of the primer pairs tested in cranberry, 89% resulted in successful amplification and 35% of those amplified polymorphic fragments among the cranberry genotypes. Of the primer pairs tested in rhododendron, 74% resulted in successful amplification and 72% of those amplified polymorphic fragments among the rhododendron genotypes. Thus, these markers should be useful for DNA fingerprinting, mapping and assessing genetic diversity within cranberry and rhododendron species, in addition to blueberry species.

Fingerprinting and analysis of genetic relationships. RAPD markers have been used for DNA fingerprinting representative selections and cultivars of the three major commercially grown types of blueberries, i.e. the highbush, lowbush and rabbiteye types. EST-PCR markers and CAPS markers derived from EST-PCR markers have been used for DNA fingerprinting representative selections and cultivars of mainly highbush blueberry types but also including a couple of rabbiteye and one lowbush *V. darrowi* genotype. In addition, RAPD, EST-PCR and CAPS and isozyme markers have been used to assess the genetic relationships of various blueberry populations, selections and cultivars.

Camp (1945) recognized nine diploid species in his taxonomic treatment of the

Cyanococcus section, as did Galletta (1975). Later, Vander Kloet (1988) recognized six species, grouping the two lowbush hillside blueberries, *V. pallidum* and *V. vacillans*, into a single taxon, *V. pallidum*, and the three highbush blueberries, *V. atrococcum*, *V. caesariense* and *V. elliotii*, into a single highbush taxon, *V. corymbosum*. These treatments were based primarily upon morphology. Bruederle and Vorsa (1994) employed allozyme data, collected at 11 polymorphic loci, to assess the genetic relationships of representative diploid blueberry populations. Genetic similarity values were calculated and a cluster analysis was performed on the similarity data matrix. The allozyme data did not refute the reduction of the two hillside blueberries *V. pallidum* and *V. vacillans* to *V. pallidum* as proposed by Vander Kloet (1978), but did support the recognition of two highbush taxa, *V. corymbosum* and *V. elliotii*, instead of only *V. corymbosum*. These results support the recognition of the seven diploid species described in the Introduction: two highbush species, *V. corymbosum* and *V. elliotii*, and five lowbush species, *V. boreale*, *V. darrowi*, *V. myrtilloides*, *V. pallidum* and *V. tenellum*.

Most commercially important improved rabbiteye (*V. ashei*) cultivars were developed from only four original native selections from the wild (Aruna *et al.*, 1993). Because of this very narrow germplasm base, Aruna *et al.* (1993) used RAPD markers generated from amplification with 20 RAPD primers to investigate the extent of genetic relatedness among 19 cultivars of rabbiteye blueberry, 15 improved cultivars and the four original selections from the wild. As expected, results showed that all the improved cultivars are progressing towards increased genetic similarity when compared with the initial four wild selections. In addition, clustering of genotypes based on genetic similarity estimates generally agreed with known pedigree information, grouping siblings with each other and with one or both parents. In an extension of this original study, Aruna *et al.* (1995) developed a cultivar key for distinguishing the 19 rabbiteye cultivars based on 11 RAPD markers amplified from four RAPD primers.

Levi and Rowland (1997) used RAPD and ISSR markers to differentiate and evaluate genetic relationships among 15 highbush (*V. corymbosum*) or highbush hybrid cultivars, two rabbiteye (*V. ashei*) cultivars and one southern lowbush (*V. darrowi*) selection from the wild. Fifteen RAPD and three ISSR markers were chosen to construct a DNA fingerprinting table to distinguish among the genotypes in the study. A cluster analysis, based on similarity coefficients calculated from the molecular marker data, effectively separated out the different species examined. However, clustering of genotypes within the *V. corymbosum* group did not agree well with known pedigree data. The authors cautioned against using RAPD or ISSR marker data alone to assess genetic relationships of cultivars or selections within a species. Arce-Johnson *et al.* (2002) reported using two RAPD primers to distinguish five highbush Chilean cultivars.

Burgher *et al.* (1998) screened 26 wild lowbush (*V. angustifolium*) clones, including six named cultivars and 12 selections, with 30 RAPD primers. All could be differentiated using 11 of the primers. Clustering of genotypes correlated fairly well with geographic origin of the clones.

Most recently, Rowland *et al.* (2003a) used EST-PCR and EST-PCR-derived CAPS markers to differentiate and evaluate genetic relationships among 15 highbush (*V. corymbosum*) or highbush hybrid cultivars, two rabbiteye (*V. ashei*) cultivars and two wild selections (one *V. darrowi* and one diploid *V. corymbosum* selection), which are the original parents of a mapping population. A subset of four EST-PCR primer pairs was identified that were sufficient to distinguish all the genotypes. As with the earlier RAPD marker data, a fair, but not strong, correlation between the similarity coefficients calculated from molecular marker data and coefficients of co-ancestry calculated from pedigree information was found.

Besides DNA fingerprinting and analysis of genetic relationships in blueberry, RAPD markers have also been used to examine the mode of inheritance in an interspecific hybrid of *V. darrowi* and *V. corymbosum* (Qu and Hancock, 1995) and to determine the mode of

2n gamete formation in a *V. darrowi* breeding selection (Qu and Hancock, 1995; Vorsa and Rowland, 1997). Results indicated that the mode of inheritance in the hybrid is tetrasomic (Qu and Hancock, 1995). Results from the 2n gamete work were consistent with the operation of predominantly first division restitution mechanisms of 2n gamete formation (Qu and Hancock, 1995; Vorsa and Rowland, 1997), but suggested that second division restitution mechanisms are operating as well (Vorsa and Rowland, 1997).

Mapping. Efforts are currently under way to develop genetic linkage maps for blueberry that are saturated enough to map QTLs controlling chilling requirement, cold hardiness, heat tolerance and fruit quality. Initial (relatively low density) maps have been constructed using primarily RAPD markers for four blueberry populations, three diploid populations and one tetraploid population. Rowland and Levi (1994) reported construction of the first RAPD-based genetic linkage map of blueberry using a diploid population segregating for chilling requirement, which resulted from a test cross between an F_1 interspecific hybrid (*V. darrowi* $\times$ *V. elliotii*) and another *V. darrowi* clone. A test cross was used because diploid blueberry species are essentially self-sterile, tolerating little inbreeding; therefore, true F_2 or back-crosses cannot be easily generated for mapping. The map currently comprises 72 RAPD markers mapped to 12 linkage groups, in agreement with the basic chromosome number for blueberry. Since that initial map was developed, Rowland *et al.* (1999) have focused on construction of RAPD-based genetic linkage maps using diploid blueberry populations shown to be segregating for both chilling requirement and cold hardiness. The populations resulted from test crosses between F_1 interspecific hybrids, *V. darrowi* $\times$ diploid *V. corymbosum*, and another *V. darrowi* clone and another diploid *V. corymbosum* clone. Most recently, a few EST-PCR markers have been added to these maps; the map of the *V. corymbosum* test cross currently comprises approximately 90 RAPD and EST-PCR markers and the map of the *V. darrowi* test cross comprises approximately 70 RAPD and EST-PCR mark-

ers (Rowland *et al.*, 2003c; L.J. Rowland, unpublished). In addition, large portions of both populations have been evaluated for chilling requirement and cold hardiness and a generation means analysis has been used to study the inheritance of cold hardiness in the cold-acclimatized state (Arora *et al.*, 2000). Results from the generation means analysis indicated that the cold hardiness data best fitted a simple additive-dominance model of gene action (a model in which the genes controlling cold tolerance are assumed to have simple additive and dominance effects). Furthermore, in this study, the magnitude of the additive gene effect was greater than that of the dominance gene effect. A preliminary QTL analysis using the current genetic linkage map and cold hardiness data for the *V. corymbosum* test cross population has identified one putative QTL associated with cold hardiness that explains ~20% of the genotypic variance (Rowland *et al.*, 2003c).

Qu and Hancock (1997) reported construction of an RAPD-based genetic linkage map of a tetraploid blueberry population that should be segregating for high fruit quality, heat tolerance and cold tolerance. The population resulted from a cross of US75 (a tetraploid hybrid of a diploid *V. darrowi* selection Fla 4B and tetraploid *V. corymbosum* 'Bluecrop') and another *V. corymbosum* 'Bluetta'. One hundred and forty RAPD markers unique to Fla 4B that segregated 1:1 in the tetraploid population were mapped into 29 linkage groups. The map is essentially of *V. darrowi* because US75 was produced via a 2n gamete from Fla 4B and only unique markers for Fla 4B were used (Qu and Hancock, 1997). Interestingly, it is the same *V. darrowi* selection Fla 4B that was used as the original parent plant of the diploid mapping populations described earlier (Rowland and Levi, 1994; Rowland *et al.*, 1999). Fla 4B and its hybrids, such as US75, have been used extensively in blueberry breeding programmes to develop low-chilling southern highbush cultivars.

2.1.3. Functional genomics

To gain a better understanding of changes in gene expression associated with cold

acclimatization in blueberry, Dhanaraj *et al.* (2004) have undertaken a genomics approach based on the analysis of ESTs. Two cDNA libraries were constructed using RNA from cold-acclimatized and non-acclimatized floral buds of the blueberry cultivar 'Bluecrop' and about 600 5'-end ESTs were generated from each of the libraries. About 100 3'-end ESTs were generated from the cold-acclimatized library as well. The ~1300 ESTs were deposited into GenBank, making this the first publicly available EST database for *Vaccinium*, and were assigned consecutive accession numbers beginning with CF810419. Putative functions were assigned to 57% of the cDNAs that yielded high-quality sequences based on homology to other genes/ESTs from GenBank, and these were classified into 14 functional categories. From a contiguous sequence (contig) analysis, which clustered sequences derived from the same or very similar genes, 430 and 483 unique transcripts were identified from the cold-acclimatized and non-acclimatized libraries, respectively. Of the total unique transcripts, only 4.3% were shared between the libraries, suggesting marked differences in the genes expressed under the two conditions. The most highly abundant cDNAs that were picked many more times from one library than from the other were identified as representing potentially differentially expressed transcripts. Northern analyses were performed to examine expression of eight selected transcripts and seven of these were confirmed to be preferentially expressed under either cold-acclimatizing or non-acclimatizing conditions. Only one of the seven transcripts, encoding a dehydrin, had been found previously to be up-regulated during cold acclimatization of blueberry (Levi *et al.*, 1999). Messages encoding a senescence-associated protein, an early light-inducible protein, beta amylase, and an unknown protein were found to be up-regulated during cold acclimatization. Messages encoding histone protein H3.2 and BURP-domain dehydration-responsive protein RD 22 were found to be down-regulated during cold acclimatization. This study has demonstrated that analysis of ESTs is an effective strategy to identify candidate cold acclimati-

zation-responsive transcripts in blueberry. In addition, the set of unique blueberry cDNAs is available for future microarray analyses.

3. Micropropagation

Shoot tip propagation of blueberry was initiated by Lyrene (1978), who reported successful *in vitro* propagation of rabbiteye (*V. ashei*) blueberry seedlings. Since then, several reviews (Smagula and Lyrene, 1984; George *et al.*, 1987; Zimmerman, 1991; Galletta and Ballington, 1996) have summarized the *in vitro* technology for blueberry propagation. Interest in *in vitro* techniques resulted from the need to rapidly produce plants from breeding populations for field trials and/or to provide sufficient numbers of plants of a newly released cultivar.

The first studies conducted on rabbiteye blueberry (Lyrene, 1978, 1980, 1981) reported a 50-fold multiplication rate every 4 months with some clones on Knop's medium (Knop, 1865) containing 6(γ -dimethylallylamino)-purine (2-isopentenyladenine) (2iP) at 75 μ M, and shoots rooted easily in a peat-perlite medium under mist in a greenhouse. Best success was achieved with juvenile material, and shoot culture propagation was achieved for 'Beckyblue' and 'Bluebelle'. Rooting of cuttings taken directly from tissue culture or from 3-month-old tissue culture-derived plants averaged 95% for 'Beckyblue' and 96% for 'Bluebelle' after 19 days compared to 7% for 'Beckyblue' and 0% for 'Bluebelle' for softwood cuttings from field-grown plants. These data suggested that easy-to-root blueberry cuttings could be obtained by exploiting reversion to juvenility in tissue culture. Studies by Young and Cameron (1985a,b) determined that maximum shoot formation occurred under continuous light conditions (30 μ mol/m²/s), and they recommended Lloyd and McCown's (1980) mineral salts supplemented with 1 g/l casein hydrolysate and 5 μ M 2iP for rapid clonal propagation.

The first studies on shoot tip propagation of lowbush blueberry (*V. angustifolium*) were conducted by Frett and Smagula (1983), who reported that the greatest number of usable

shoots for subculturing occurred on Zimmerman medium (Zimmerman and Broome, 1980) supplemented with 100 μ M 2iP and 5.7 μ M indole-3-acetic acid (IAA). Transferring these shoots to medium with 59 μ M 2iP produced the best rooting response (44%). Rooting was increased to 98% if, during the last subculture, shoots (> 20 mm in length) were transferred to a medium with 24.6 μ M 2iP and incubated under a light intensity of 140 μ mol/m²/s (Brisette *et al.*, 1990).

Smagula and Litten (1989) and Litten *et al.* (1992) studied mycorrhizal inoculation of lowbush blueberry as an aid to micropropagation. Smagula and Litten (1989) found no growth stimulation of already rooted microcuttings in substrate fortified with a suspension of *Hymenoscyphus ericae*; however, when Litten *et al.* (1992) rooted shoots in the presence of either *H. ericae* or *Scytalidium vaccinii*, they reported that, both in irradiated peat mix inoculated with *S. vaccinii* and in unirradiated peat mix with *H. ericae*, microcuttings grew taller and branched more than with irradiated or unirradiated, uninoculated treatments.

Because incomplete coverage of young fields is typical for lowbush blueberry, studies were conducted to compare the spread of blueberry by rhizome formation from *in vitro*-propagated plants and rooted softwood cuttings (Morrison *et al.*, 2000). After 2 years of field growth, micropropagated plantlets rooted after three subcultures averaged 20 rhizomes as compared with six rhizomes for rooted softwood cuttings.

The earliest studies on shoot tip propagation of highbush blueberry (*V. corymbosum*) date back to 1979–1980 (Cohen and Elliott, 1979; Cohen, 1980; Zimmerman and Broome, 1980). Cohen and Elliott (1979) and Cohen (1980) employed actively growing blueberry shoot tips, and quarter- to half-strength Murashige and Skoog (1962) (MS) salts with 25 μ M 2iP. A multiplication rate of six- to eightfold every 2 weeks was achieved for several cultivars: 'Atlantic', 'Jersey', 'Dixie', 'Stanley', 'Burlington', 'Darrow', 'Berkeley', 'Ivanhoe', 'Blueray', 'Bluecrop' and 'Earliblue'. Similar studies were conducted by Zimmerman and Broome (1980), who

used a modified Anderson medium (Anderson, 1975) containing 75 μM 2iP. With this high level of 2iP, the shoots produced had extremely short internodes and some subsequent studies revealed that the number of new shoots produced was directly related, but the length of internodes was inversely related, to the 2iP concentration. Transferring clumps of shoots from a medium with high 2iP to one with a low level (5 to 25 μM) permitted the existing shoots to elongate sufficiently to be rooted. Rooting was accomplished by dipping the bases of cuttings in 1000 mg/l indole-3-butyric acid (IBA) in 50% ethanol, and inserting the cuttings into milled sphagnum in plastic flats that were placed under intermittent mist in the greenhouse.

Wolfe *et al.* (1983) conducted studies to compare various media and to determine the optimum medium for micropropagating highbush 'Bluecrop'. The highest number of shoots 10 mm or longer (1.5) and the greatest number of secondary shoots (2.6) were produced on woody plant medium (WPM) (Lloyd and McCown, 1980). Maximum % rooting occurred with cuttings > 20 mm. Orlikowska (1986) was able to increase 'Bluecrop' shoot production to 6.8 shoots from one explant when shoots were first incubated in the dark at 4°C for 4 to 6 weeks on medium with 75 μM 2iP and then transferred to Zimmerman's medium (Zimmerman and Broome, 1980) with the same concentration of 2iP, and incubated at 25°C under a light intensity of 6 $\mu\text{mol}/\text{m}^2/\text{s}$.

Grout and Read (1986) studied the influence of the stock plant propagation method on propagation and rooting of halfhush blueberry 'Northblue'. Microshoots from tissue culture-derived stock plants produced approx. fourfold more shoots and rooted better than shoots from standard-derived stocks after 12–15 weeks of culture on WPM (pH 5.2).

Chandler and Draper (1986) investigated the use of 6-(4-hydroxy-3-methylbut-2-enylamino)purine (zeatin) for the propagation of highbush blueberries. They found that 18–37 μM zeatin induced proliferation of two to four times as many shoots as 2iP. Eccher and Noe (1989) found that 22.8 μM

zeatin was less phytotoxic than 2iP and could induce greater numbers of sprouted explants, thus being useful for the early stages of micropropagation. Tests with 12 highbush blueberry genotypes showed significantly higher rates of new shoot growth on modified WPM with 18.3 μM zeatin than with either 50 or 75 μM 2iP (Reed and Abdelnour-Esquivel, 1991), and shoot initiation under low light (10 $\mu\text{mol}/\text{m}^2/\text{s}$) and 25°C was superior to that at 4°C in darkness. Under these optimum conditions, Reed and Abdelnour-Esquivel (1991) reported that initiation rates > 60% were achieved for 89 of 96 blueberry accessions tested.

To facilitate commercial disease-free plant production in the Mediterranean area, Gonzalez *et al.* (2000) initiated studies to develop a uniform method of micropropagation using nodal segments from mature field-grown highbush blueberry plants. Cultures from lateral shoots of hardwood cuttings and nodal segments of softwood cuttings were successfully established on WPM with 18 μM zeatin and were multiplied on WPM with 25 μM 2iP.

Only a few studies (Noè and Eccher, 1994; Noè *et al.*, 1998) have investigated the influence of irradiance on *in vitro* growth and proliferation of highbush blueberry. Variations in photosynthetic photon flux density (PPFD) were shown to influence proliferation and growth, which were highest at 55 $\mu\text{mol}/\text{m}^2/\text{s}$ and decreased at higher PPFD (Noè and Eccher, 1994). Noè *et al.* (1998) also determined that a very low blue/red ratio (no transmission at wavelengths shorter than 520 nm) strongly promoted axillary shoot production and elongation. Baraldi *et al.* (1988) have suggested that a low blue/red ratio promotes cytokinin synthesis and thus increases axillary shoot activity in *Prunus* GF 655–2.

Most recently, in an effort to increase highbush blueberry shoot production *in vitro* without negatively impacting subsequent genetic engineering experiments, Cao *et al.* (2003) investigated the effects of sucrose concentration in the propagation medium on shoot proliferation and on the transfer of an intron-containing glucuronidase (GUS) gene into leaf explants from the propagated

shoots of cultivars 'Bluecrop', 'Duke' and 'Georgiagem'. These studies demonstrated that shoot pre-treatments with sucrose can negatively influence subsequent gene delivery into blueberry leaf explants and thus a shoot multiplication medium containing 15–29 mM sucrose was recommended to promote axillary shoot proliferation without interfering with gene delivery.

Rooting and establishment of *in vitro* blueberry plantlets in the presence of mycorrhizal fungi was studied (Lareau, 1985) because the presence of mycorrhiza has been shown to stimulate maximum development of blueberry plants and fruit production (Powell and Bates, 1981) and enhance the N and P supply in cranberry (*V. macrocarpon*) plants (Read, 1983). Lareau (1985) reported that unrooted blueberry cuttings could be inoculated with mycorrhizae *in vitro* and, although there was no direct, positive effect of these fungi on rooting or early growth of plantlets, better growth of mycorrhizal plants could be expected following transplantation.

Rooting blueberry microcuttings *ex vitro* can reduce costs (Zimmerman, 1987); thus, Isutsa *et al.* (1994) conducted investigations to identify environmental conditions that would accelerate rooting and acclimatization and improve survival of *ex vitro* blueberry microcuttings. They developed a protocol that enables production of field-sized blueberry transplants within 6 months of obtaining shoots from tissue culture. The protocol involves subjecting *in vitro* shoots to *ex vitro* rooting in a fog chamber under a photosynthetic photon flux (PPF) of 100 $\mu\text{mol}/\text{m}^2/\text{s}$ for 7 weeks, transferring plants to a fog tunnel for 2 weeks and then to a greenhouse for 7 more weeks. Plant survival and rooting of highbush 'Berkeley' and halfhigh 'Northsky' were almost 100% under these conditions; within 6 months, these plants were 30–60 cm and suitable for field planting.

Long-term effects of *in vitro* propagation of 'Northblue' halfhigh blueberry under greenhouse and field conditions have been reported (Grout *et al.*, 1986; El-Shiekh *et al.*, 1996) because micropropagation of several fruit species has resulted in changes in growth habit (Swartz *et al.*, 1981, 1983).

Grout *et al.* (1986) reported that 'Northblue' plants propagated *in vitro* had two to three times more basal branches at 27–34 weeks after propagation compared to cutting-propagated plants. At 82 weeks after propagation and under field conditions, the *in vitro* plants produced twice the number of flower buds as the number on cutting-propagated plants. El-Shiekh *et al.* (1996) observed these same plants from 3 to 10 years after the initial planting to determine the persistence of increased fruit yield effects observed during the initial 3 years after planting (Read *et al.*, 1989), and reported that the effect of propagation method on yield and growth habit is limited to early years in warmer locations, but can be of longer-term significance in colder areas with shorter growing seasons and lower winter temperatures. In contrast, yield in the first 3 years was similar for *in vitro*- and cutting-propagated plants of 'Bluecrop'; however, in the fourth and fifth year, cutting-propagated plants fruited better, bearing fruit significantly larger than *in vitro* plants (Smolarz and Chiebowska, 1997).

4. Somatic Cell Genetics

4.1. Regeneration

4.1.1. Organogenesis

Early studies to induce organogenesis yielded callus from stem internodes of lowbush blueberry on MS medium with 0.5–2.3 μM 2,4-dichlorophenoxyacetic acid (2,4-D) (Nickerson and Hall, 1976). Nickerson (1978) was able to induce shoots from cotyledons and hypocotyl sections on Anderson's medium containing 23 μM IAA and 75 μM 2iP, and initiated roots from pericarp callus on Nitsch's (1965) medium with L-tryptophan at 100 mg/l and 0.47 μM kinetin (Nickerson, 1980). High-frequency shoot formation (43 shoots per intact explant) from leaves of a *V. corymbosum* $\times$ *V. ellioti* cross was obtained on Knop's medium containing 24.6 μM 2iP (Dweikat and Lyrene, 1988). Shoot production did not increase if the 2iP concentration was increased.

The first success with organogenesis from highbush blueberry (Billings *et al.*, 1988) occurred from leaf explants of *in vitro*-propagated shoots. Seven and 11 shoots per regenerating leaf segment were produced from 'Berkeley' and 'Bluehaven' blueberry, respectively, on WPM with 15–20 μM 2iP. A maximum of one shoot per leaf explant from 'Bluecrop' was produced on MS medium with 50–100 μM 2iP (Callow *et al.*, 1989). Rowland and Ogden (1992, 1993) investigated zeatin riboside (ZR) for highbush blueberry regeneration from leaf explants. ZR was more effective than either 2iP or zeatin for regeneration of 'Sunrise', with maximum shoot production (21 shoots per explant) on modified WPM with 20 μM ZR. Unfortunately, commercially important 'Duke' and 'Bluecrop' did not produce any shoots on any of the media tested. Hruskoci and Read (1993) compared shoot regeneration from internode explants on Zimmerman's medium with either 25 μM zeatin or 25 μM thidiazuron (TDZ). Maximum regeneration occurred when the top two (youngest) internode segments were cultured on zeatin.

Cao and Hammerschlag (2000) studied the effects of ZR and TDZ, PPF, length of dark period and age of explant on shoot regeneration from leaves of several commercially important blueberry cultivars.

Maximum regeneration occurred on 20 μM ZR under a PPF of $55 \pm 5 \mu\text{mol}/\text{m}^2/\text{s}$. 'Duke' regenerated equally well on ZR and 1 μM TDZ, and all cultivars ('Duke', 'Georgiagem', 'Sierra' and 'Jersey') except 'Bluecrop' regenerated shoots. Cao *et al.* (2002) also reported that growth regulator pre-treatments enhance shoot organogenesis from leaf explants of 'Bluecrop'. A maximum of 98% shoot regeneration and 11 shoots regenerating per leaf explant occurred when explants of 2-week-old shoot cultures were incubated on pre-treatment medium no. 1 containing 5 μM TDZ and 2.6 μM naphthaleneacetic acid (NAA) for 4 days, next on pre-treatment medium no. 2 containing 7 μM ZR and 2.6 μM NAA for 3 days, then on regeneration medium containing 1 μM TDZ for 6 weeks, and finally on medium without growth regulators for 10 days (Fig. 8.1.2).

4.2. Genetic manipulation

4.2.1. Mutation induction and somaclonal variation

Because the basic blueberry chromosome number is 12, blueberry species (diploids, tetraploids and hexaploids) range in chromosome number from 24 to 72 and have a tremendous range in habitat preferences.

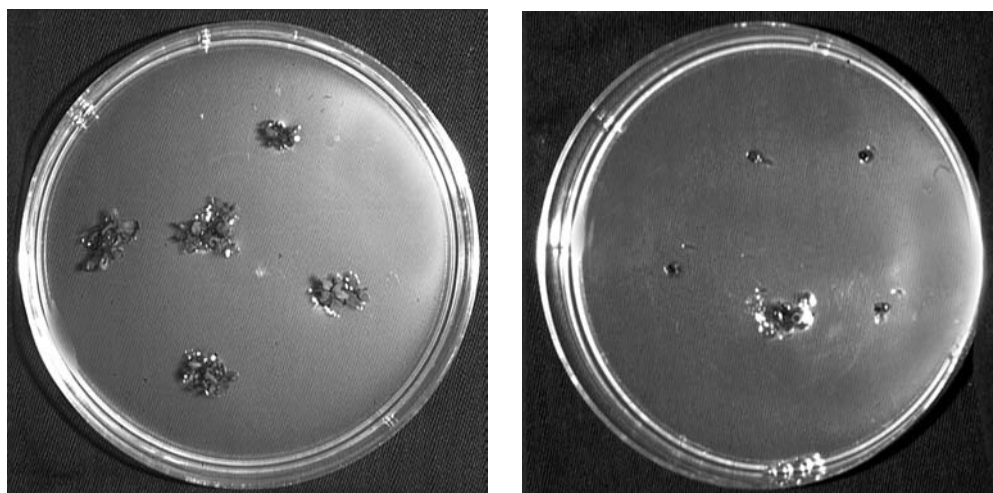


Fig. 8.1.2. Shoot formation from 'Bluecrop' leaf explants with pre-treatment (left) or without pre-treatment (right).

Hybridization between certain species has been difficult to achieve due to chromosome number differences and the inability to easily induce polyploids (Moore *et al.*, 1964; Dweikat and Lyrene, 1989). *In vitro* plant tissue culture has been shown as a method for inducing and screening for desirable mutations (Murashige and Nakano, 1966; Skirvin, 1978; Larkin and Scowcroft, 1981; Hammerschlag, 1992). Lyrene and Perry (1982) reported that a combination of colchicine treatments with blueberry tissue culture facilitates chromosome doubling. Although several methods were successfully used for polyploid induction, their preferred method was to transfer three-node segments from rapidly growing shoot tip cultures to vials of modified Knop's liquid medium containing 0.2% colchicine. The vials were placed on a rotating wheel for 24 h, rinsed and placed on Knop's medium with 25 μ M 2iP. The advantage of the *in vitro* method for blueberry was that polyploidy could be screened for, based on increase in stem diameter. This method or similar methods have been used to induce tetraploids in *V. darrowi*, *V. elliotii* and *V. darrowi* $\times$ *V. elliotii* hybrids (Perry and Lyrene, 1984), 8x plants from 4x *V. corymbosum* clones (Goldy and Lyrene, 1984) and 6x plants from a triploid (*V. corymbosum* (4x) $\times$ *V. elliotii* (2x)) hybrid (Dweikat and Lyrene, 1989). Induction of these hybrid hexaploids facilitated crossing with hexaploid *V. ashei* (this was over 300 times higher than the number produced by the original highly sterile triploid) and the induction of tetraploid *V. elliotii* facilitated crossing with cultivated highbush blueberry (Dweikat and Lyrene, 1991).

Commercial blueberry production is limited to soils with inherently low pH or that have been treated with acidifying soil amendments (Ballinger, 1966; Chandler *et al.*, 1985). Chandler *et al.* (1985) suggested that cultivars adapted to upland mineral soils should be tolerant of lower organic matter, higher pH and lower average moisture content and that the initial screen for upland soil adaptability be done in the greenhouse or in a place where soil conditions could be carefully controlled. *In vitro* systems provide a more highly controlled environment than field or greenhouse environments and have

been used to study whether blueberry growth is affected by pH (Wolfe *et al.*, 1986). Thus, seeds from 64 crosses among eight highbush, lowbush and *V. corymbosum*/*V. angustifolium* hybrid-derived parents were evaluated *in vitro* on a modified Zimmerman's medium at low (5.0) and high (6.0) pH (Finn *et al.*, 1991). Plants were grown for 21 weeks and then data were collected on plant survival and growth traits. The results indicated that *in vitro* screening in concert with a traditional breeding programme should be effective in improving blueberry tolerance to higher pH.

4.2.2. Genetic transformation

Blueberry is an especially suitable target for improvement via direct gene manipulations because of the genetic limitations associated with high heterozygosity and polyploidy, which hamper improvement through traditional breeding methods. Because natural infection of blueberry by *Agrobacterium tumefaciens* is rare (Demaree and Smith, 1952) and the possible resistance to this bacterium could limit its usefulness for transformation, studies were conducted to investigate the susceptibility of blueberry to several *A. tumefaciens* strains (Rowland, 1990). Groups of six to eight 6-month-old seedlings were each inoculated with *A. tumefaciens* strains T37, C58, A281, A518 and B6. Gall formation occurred in response to infection with strains A281, T37 and C58, indicating that *A. tumefaciens* could be used for transformation.

Hancock *et al.* (1990) conducted transformation studies with the highbush 'Sierra' with *A. tumefaciens* strain LBA4404 containing the plasmid pBI121.1 carrying the neomycin phosphotransferase gene (NPTII) and the gene for β -glucuronidase (GUS). They investigated the effects of concentration of *A. tumefaciens*, length of co-cultivation and antibiotic treatments on transformation. Inoculations of leaf tissue with *A. tumefaciens* for longer than 30 and incubation periods of > 32 h resulted in uncontrollable proliferation of the bacteria. Also, raising the level of the antibiotic cefotaxime to 250 or 500 mg/l inhibited shoot regeneration. Thirteen putative transgenics were recovered following placement on solid

antibiotic medium with cefotaxime at 100 mg/l and incubation at 20°C. Preliminary Southern blot analyses suggested stable transformation for three out of 13 putative transgenics. However, because these putative transgenics were subsequently lost due to a growth chamber failure, research with these plants could not be continued (J. Hancock, personal communication).

Rowland and Ogden (1993) initiated transformation studies with *A. tumefaciens* strain C58C1/pGA482. Leaf discs of highbush blueberry 'Sunrise' were dipped in a bacterium suspension, co-cultivated on modified WPM without antibiotics or growth regulators for 2 days and then transferred to modified WPM medium with 20 µM ZR, 500 mg cefotaxime/l and 10 mg kanamycin/l. Although shoots were recovered, they were not truly transformed as determined by PCR and Southern blot data.

Graham *et al.* (1996) reported transformation of blueberry halfhigh 'North Country' using disarmed *A. tumefaciens* strain LBA4404 containing a binary vector with an intron-containing GUS marker gene (Vancanneyt *et al.*, 1990). Explants from 3-week-old shoot cultures were infected for 20 min, co-cultivated for 2 days and then transferred to WPM containing 14.8 µM 6-γγ-dimethylallylaminopurine (DMAAP). Plants were regenerated in the absence of antibiotics. Although regenerants were GUS-positive, Southern analysis was not conducted to confirm transformation.

Cao *et al.* (1998) conducted an in-depth study on factors that influence the early stages of transformation. They used ten highbush blueberry cultivars and disarmed *A. tumefaciens* strains LBA4404 (pAL4404) (Hoekema *et al.*, 1983) (slightly virulent) and EHA105 (pEHA105) (Hood *et al.*, 1993) (highly virulent), both containing the binary vector p35SGUSint. Explants from 1- to 3-week-old shoot cultures were incubated with *A. tumefaciens* (gyratory shaker, 40 rpm) for 2 h and then co-cultivated for 2–5 days on filter paper saturated with co-cultivation medium containing: N6 macro salts (Chu *et al.*, 1975), Linsmaier and Skoog micro salts (Linsmaier and Skoog, 1965), 0.56 µM myo-inositol, 3 µM thiamine HCl, 20 µM acetosy-

ringone, 5 µM TDZ, 1.1 µM NAA, 87.6 mM sucrose and 300 mg/l casein hydrolysate. After co-cultivation, explants were assayed immediately for GUS activity or transferred to a callus-inducing medium for 2 weeks and then assayed. Four days of co-cultivation with strain EHA105 yielded 50-fold more GUS-expressing zones than 2 days, and GUS expression following infection with strain LBA4404 was not observed until 4 days after co-cultivation. Significant differences among cultivars were observed for both GUS-expressing leaf zones and calluses. For some cultivars, explant age influenced the number of GUS-expressing leaf zones and calluses. These results suggest that difficulty in obtaining stable transformation in previous studies (Rowland and Ogden, 1993; Graham *et al.*, 1996) could have been due to co-cultivation for only 2 days with the less virulent strains C58C1 and LBA4404.

4.3. Cryopreservation

Apical meristems of shoot tip and nodal cultures of *Vaccinium* species (*V. corymbosum*, *V. uliginosum* L. and *V. ovatum* Pursh) were tested for their ability to be cryopreserved following cold hardening (Reed, 1989). *In vitro*-grown plants were cold hardened/chilled at alternating temperatures (22°C day/–1°C night) for 0, 1, 3, 5 or 7 weeks before the meristems were excised and cooled to –35°C at four different rates. Results indicated that survival rate varied greatly among species. Whereas *V. corymbosum* survival increased almost sixfold following 3 or more weeks of cold hardening/chilling, *V. ovatum* did not improve significantly under any treatment. Warm-grown plants of *V. corymbosum* survived only at the 0.1°C/min freezing rate while those of the other species did not survive at any rate.

5. Conclusions

The domestication of the wild blueberry has occurred entirely within the 20th century, and great progress has been made during this time using traditional breeding approaches.

Initially, utilizing a narrow genetic base and only a few generations of hybridization and selection, cultivars were developed with major increases in fruit size, fruit quality, productivity, disease resistance and bush adaptation (Galletta and Ballington, 1996). In the last 35 years, significant advances have also been made to broaden the germplasm base in highbush blueberries, utilizing wild $\times$ cultivated highbush crosses and a variety of homoploid and heteroploid interspecific crosses (Ballington, 2001).

With respect to molecular genetic research in blueberry, molecular markers have been developed for DNA fingerprinting, analysis of genetic relationships and mapping, several cDNA and genomic clones have been isolated and sequenced and an EST database has been made publicly available. The types of markers currently available include isozymes, RFLP, RAPD, ISSR, EST-PCR and EST-PCR-derived CAPS markers. Molecular markers have been identified that are useful for DNA fingerprinting representative selections and cultivars of the three major commercially grown types of blueberries: the highbush, lowbush and rabbiteye types. Initial (relatively low density), primarily RAPD-based, genetic linkage maps have been developed for three diploid and one tetraploid blueberry populations. More EST-PCR markers are being developed from cDNA libraries prepared from RNA from flower buds of cold-acclimatized and non-acclimatized blueberry plants and they are being added to the current genetic linkage maps. With further saturation, these maps and segregating populations should allow researchers to map QTLs controlling such traits as chilling requirement, cold hardiness, heat tolerance and fruit quality. To date, only one putative QTL associated with cold hardiness has been identified in one of the mapping populations. Thus far, all efforts to clone

genes from blueberry have followed from the identification and characterization of cold hardiness-associated proteins. Using traditional molecular genetic approaches, one full-length cDNA clone encoding the 60 kDa dehydrin from blueberry and several partial-length dehydrin cDNA and genomic clones have been isolated and sequenced. Most recently, a genomic approach based on the analysis of ESTs has resulted in several newly described genes that are up- and down-regulated during cold acclimatization.

Although micropropagation is now an accepted practice for blueberry, other tissue culture-related techniques such as protoplast fusion, induced variation and gene transfer have yet to be fully developed. Most encouraging have been the recent successes to generate high-frequency shoot regeneration from leaf explants of several commercially important cultivars such as 'Duke' and 'Bluecrop' and the study of the factors that influence the early stages of transformation.

In the coming decade, we expect that traits that have been transferred to other crops with success, such as virus resistance, insect resistance and delayed ripening, will be transferred to blueberry. More genes/markers for cold and heat tolerance will probably be identified using microarray technology in blueberry and used for blueberry transformation and marker-assisted selection. We also anticipate that the nutritional value of blueberry, such as the antioxidant content, will be improved through the application of genetic engineering.

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8.2 *Vaccinium* spp. Cranberry

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1. Introduction

1.1. Botany and history

In many respects, the cranberry is a fruit that should not be a crop of any commercial significance. Although beautifully coloured, the ripe fruit is hard, very tart, subtly flavoured and, to many consumers, inedible when raw. The plant requires rather exacting growing conditions of acid peaty sands with ready access to fresh water resources and grows only 4–6 inches off the ground in extensive intertwined mats. Nevertheless, cranberries have been consumed by humans for several centuries and are now being grown as commercial crops on three continents. This success can be attributed in part to the traditions surrounding the harvest and consumption of cranberry, the ease with which cranberry combines with other fruits in processed products and the ingenuity of humans in devising unique cultivation techniques.

Cranberries belong to the *Oxycoccus* section of *Vaccinium* (family *Ericaceae*), which includes two major species, *V. macrocarpon* (Ait.) Pursh. (large-fruited or American cranberry) and *V. oxycoccus* L. (small-fruited cranberry or mossberry) (Luby *et al.*, 1990). Both species are long-lived woody, trailing, broad-leaved evergreens. The native habitat is principally in wetland areas. Flowers are

borne on 5–8 cm axillary shoots ('uprights') emerging from the nodes of the creeping vines (stolons or runners). Terminal buds on the uprights are set during the previous season and are mixed (containing both the vegetative terminal bud and the flower primordia). Bud break results in two to seven flowers emerging at the base of the new season's growth. With appropriate insect pollination, usually one to four fruit set on each upright. Both species have a relatively small genome size and a haploid chromosome number of 12. *V. oxycoccus* occurs circumboreally and can be found in regional populations that may be diploid, tetraploid or hexaploid. *V. macrocarpon* is native to the North American continent and is diploid.

1.2. Importance

Cranberry fruit was wild-collected by both native populations and settlers long before cultivation. In North America, cranberry consumption was associated historically with autumn harvest celebrations; however, the fruit was also consumed for its nutritional benefits, particularly as a winter source of vitamin C. By far the largest commercial plantings consist of *V. macrocarpon*. The earliest record of cultivation is in the

early 1800s in the eastern USA; however, other centres of cultivation emerged in the Midwest and Pacific Northwest of the USA (Wisconsin, Oregon and Washington) (Roper and Vorsa, 1997). Today, four major production areas in North America (Wisconsin, Massachusetts, New Jersey and British Columbia) produce > 90% of the world's crop. Other notable production areas include some northern states of the USA, Quebec, Canada, Chile and Poland, although new plantings are being established throughout the temperate world. Modern plantings are in highly engineered beds of up to 2 ha in size. The major commercial plantings of *V. oxycoccus* are in Russia and Eastern Europe; however, there is interest in commercializing this species in other regions (Roper and Vorsa, 1997).

1.2.1. Breeding objectives

Objectives for the genetic improvement of cranberry are much the same as for any other fruit crop (Galletta and Ballington, 1996). However, especially important to cranberry is the reduction in environmental impact on wetland and water resources of production practices (Roper and Vorsa, 1997). Thus any methodology that will either allow the use of more biorational pesticides or increase the crop's natural resistance to pests would be potentially valuable to the cranberry industry. In addition, since the major commercial use of cranberry is for juices, intense pigment development in the fruit is important. Recently, a new hybrid selection developed for intense and early fruit pigmentation has been released (McCown and Zeldin, 2003), and is the first cranberry to be patented (McCown *et al.*, 2003).

Although still relatively primitive with respect to genetic improvement, recent cranberry breeding has benefited from biotechnology. The small stature of the plant and the relative short breeding cycle (approx. 3 years from seed to seed, but with manipulation as short as 1 year (Serres *et al.*, 1994b)) complement biotechnological manipulations, making the cranberry plant a potential model for temperate fruits in general (Serres *et al.*, 1994b).

1.2.2. Breeding accomplishments

Early commercial production of cranberry relied solely on cultivars derived from native populations. Selections are readily propagated by cuttings, and the cultivars are maintained as clones. Over 100 selections from native populations have been named (Dana, 1983). The first and most significant cranberry breeding programme, which began in the late 1930s, was in the USA and released its first hybrid cultivars in the mid-20th century (Chandler *et al.*, 1950; Chandler and DeMoranville, 1961). Although six cultivars were eventually released from this programme, 'Stevens' has shown consistently high yields of moderately coloured fruit in diverse regions and thus has been the most widely planted hybrid (Roper, 2001).

Since the current cranberry cultivars are either native selections or have benefited from only one generation of controlled breeding and selection, the opportunity for major improvements in crop productivity from genetic enhancement programmes is immense. Although cultivars are self-fruitful, protandry and obligatory insect pollination promote cross-pollination and thus vigorous selections are highly heterozygous. Direct evidence for high genetic diversity and heterozygosity in cranberry species and selections comes from allozyme and random amplified polymorphic DNA (RAPD) studies (Sarracino and Vorsa, 1991; Novy and Vorsa, 1995; Bruederle *et al.*, 1996; Roper and Vorsa, 1997).

2. Molecular Genetics

Cranberry selections are clonal cultivars planted as dormant cuttings derived from mowing or pruning vines in commercial beds. Propagation sales usually involve multi-ton lots of baled vines, often transported across continental distances. In such massive propagation schemes, the chances of loss of cultivar clonal integrity are great, especially considering that some of the cultivars have been propagated in this way for > 100 years. Sources of contamination include a mixed original planting of the mother bed, the establishment of seedlings among the clonal cultivar or the accumulation of ran-

dom mutants in the original mother bed. Using RAPD fingerprinting, Novy and Vorsa (1995) determined that each of the four cultivars that were studied existed in commercial beds as germplasm mixes. Rather than being represented by one genotype, each cultivar was represented by multiple, often unrelated, genotypes; however, RAPD data also indicated that a predominant genotype could be discerned for each cultivar. Of 66 polymorphic bands, cultivars differed by an average of 22 bands (Novy *et al.*, 1994). Such information would be useful if cultivar integrity for new plantings were deemed a grower priority. This may be the case for new introductions, most of which may be patented and thus can carry legal and cost implications for maintaining genotype purity.

In order to overcome some of the limitations of RAPDs for fingerprinting of cranberry, Polashock and Vorsa (2002) have developed the use of sequence-characterized amplified regions (SCARs) for cranberry germplasm analyses. Separation of closely related progeny was superior with SCARs in comparison with RAPDs; however, these methods were most useful when identifying identical or closely related genotypes since genetic similarities between germplasm accessions were only comparable above the 0.90 similarity coefficient.

3. Micropropagation

Like most members of the *Ericaceae*, *V. macrocarpon* is responsive *in vitro* (Lloyd and McCown, 1981; McCown and Lloyd, 1983; Brisette *et al.*, 1990; Marcotrigiano and McGlew, 1991; Serres *et al.*, 1992, 1994a; McCown, 2000; Qu *et al.*, 2000). In general, low-salt formulations such as woody plant medium (WPM) (Lloyd and McCown, 1981) and Anderson's rhododendron salts (Anderson, 1975) and the cytokinins 2-isopentenyladenine (2iP) or zeatin are conducive to vigorous shoot growth (McCown and Lloyd, 1983; Norton and Norton, 1985). Young shoot explants are readily surface-disinfested and shoot tips and nodes respond as initial explants in culture. Shoot cultures are generally stabilized within three to six sub-

cultures. Cytokinin (2iP) concentrations from 0.1 to 10 μM are generally effective for maintaining active shoot growth, but do not have the same strong effect on axillary branching as observed with other *Ericaceae* (McCown and Lloyd, 1983; Serres *et al.*, 1994a). Shoot cultures can be maintained indefinitely through monthly subculture of shoot tips or nodal sections under long photoperiods (16–24 h of 60 to 80 $\mu\text{mol}/\text{m}^2/\text{s}$ of fluorescent lighting) and temperatures of 22–27°C. Microcuttings root *ex vitro* under high relative humidity conditions within 1 month and acclimatize readily to glasshouse conditions. We have not observed somaclonal variations or other abnormalities as long as true axillary bud multiplication is maintained.

The cloning of cranberries is inherently uncomplicated as the plant spreads naturally and can be propagated vegetatively from stolons. Stolons root at nodes and continue to advance yearly as long as new ground is available. Thus, large clonal patches can be formed in the wild. Under cultivation, quiescent stem cuttings in the form of vines can root when disked into sand or peat early in the growing season. Commercially important viral or bacterial diseases have not been a significant problem during the last decades of production. Thus, the need for more complex cloning methods, such as micropropagation, is not obvious, especially if the higher cost of micropropagules is considered. In the USA, cranberry vines may cost \$5000 to \$12,500 per hectare at a medium planting density. The cost per hectare using micropropagules in plugs would generally be two to three times more expensive, not considering the more complex process of maintaining and planting plugs.

A potential use of micropropagation is for the rapid scale-up of newly introduced cultivars. Cranberries can be readily micropropagated (Marcotrigiano and McGlew, 1991) using standard shoot culture techniques accompanied by *ex vitro* rooting in plugs (Fig. 8.2.1). To avoid the inherent high cost of micropropagules, micropropagation is best used to provide large numbers of stock plants from which cuttings are repeatedly harvested for the lower cost scale-up of the new cultivar.

Although not well documented, the



Fig. 8.2.1. Steps in the micropropagation of cranberry. Starting at left: a shoot culture, a microcutting, a microcutting placed for rooting in a transplant plug, and, at right, a field-ready acclimatized and rooted plantlet. Note the basal lateral branches on the plantlet, most of which will develop into stolons.

movement of tons of vines harvested from producing beds can potentially contaminate the new plantings with the pests and diseases plaguing the mother bed. Micropropagation can break such a linkage by providing propagules free of important pathogens, insect pests and weeds. One case where micropropagation proved particularly successful was in the establishment of the cranberry industry in Chile (Eldon Stang, 1998, personal communication). Stock microcultures of major cultivars were imported from the USA and increased on site at a specially built laboratory in Chile. Microcuttings were rooted in plug systems and used to establish the first 200 ha of plantings. Further plantings employed vine cuttings taken from these original beds. An additional advantage of this approach was that the original shoot cultures were verified to be genetically true to type, thereby assuring that the subsequent plantings were free of the cultivar-mixing common in many of the older commercial plantings in the USA.

Beds planted with micropropagated transplants are established more rapidly than dormant vine cuttings planted at the same density (Fig. 8.2.2). Besides being

rooted when planted, micropropagated cranberries show a high tendency to form stolons, a phenomenon also observed with the rhizome production from micropropagated lowbush blueberries (Morrison *et al.*, 2000). In addition, unlike vine cuttings, the micropropagated plants do not flower during the year of planting (Fig. 8.2.2) and therefore all the plant resources are used for vegetative growth. However, normal flowering and fruiting occur in subsequent years.

4. Somatic Cell Genetics

4.1. Regeneration

4.1.1. Organogenesis

V. macrocarpon has been regenerated directly from stem and leaf sections by organogenesis (Scorza and Welker, 1988; Serres *et al.*, 1992; Serres and McCown, 1995; Marcotrigiano *et al.*, 1996; Polashock and Vorsa, 2003). With optimization, a high proportion (> 95% according to Polashock and Vorsa, 2003) of explants regenerate. The highest rates of regeneration utilize the cytokinin thidiazuron (TDZ) (Serres and McCown, 1995; Polashock and Vorsa,

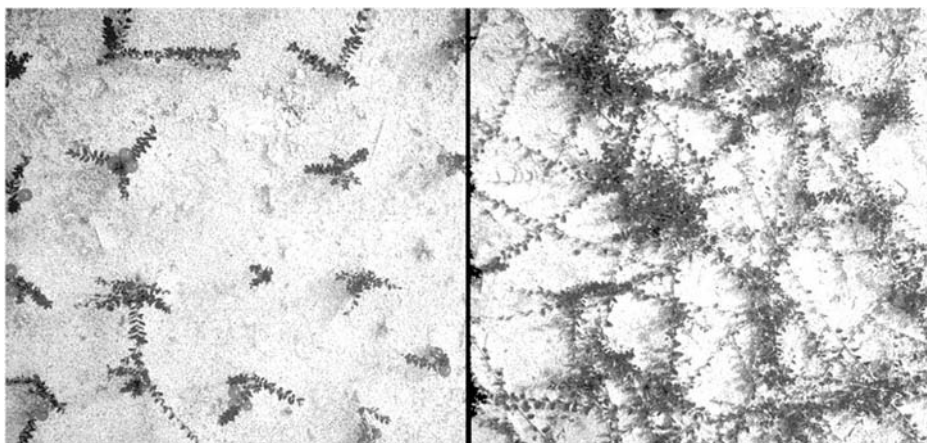


Fig. 8.2.2. First-year field plantings of cranberry from two sources: left, vine cuttings harvested in a quiescent condition from producing beds and stuck directly in the field; right, micropropagated transplants. Propagules planted in the spring and picture taken near the end of the first growing season. Although all propagules established successfully, note the more profuse stolon development from the micropropagated source. Similar stolon development occurred on the dormant cuttings during the second growing season. Also note the fruiting on some of the vine cuttings.

2003); however, although cranberry callus is readily grown (Madhavi *et al.*, 1995), regeneration from callus has not been verified and inclusion of auxins in the regeneration protocol generally reduces regeneration (Qu *et al.*, 2000; Polashock and Vorsa, 2003). For stem tissues, microshoots are defoliated and placed horizontally on WPM supplemented with 1 μ M TDZ and 10 μ M 2iP. Internodes regenerate more shoots than nodal tissues and most of the buds form along the edges of the stem at the surface of the medium (R. Serres, 1993, personal communication). Adventitious buds are visible within 14 days and well-developed shoots emerge within a month (Serres *et al.*, 1992). For leaf tissue, a medium containing 10 μ M TDZ and 5 μ M 2iP is effective. Shoots emerge from the adaxial side of the leaf and regeneration is greatest when the adaxial side is in contact with the medium. In all cases, elongation of the adventitious shoots is facilitated by subculture to a medium with low (0.1 μ M) or no cytokinins. All cultivars so far tested and reported have regenerated, although differences in rates of regeneration between cultivars exist (Qu *et al.*, 2000; Polashock and Vorsa, 2003). No stable somaclonal variants from organogenic cultures have been documented.

4.1.2. Ploidy manipulation

Although native tetraploid and hexaploid populations of *V. oxycoccus* are prevalent, polyploid populations of *V. macrocarpon* have not been described. The diploid forms of *V. oxycoccus* occupy the harsher and more northerly regions of the distribution of this species. The natural *V. oxycoccus* polyploids are probably autopolyploids, although allopolyploidy has also been suggested (Vander Kloet, 1988). There are commercial varieties of both the diploid and tetraploid forms of *V. oxycoccus*.

Artificial tetraploids of *V. macrocarpon* were developed in the 1940s, using a colchicine treatment on stolon axillary buds (Derman and Bain, 1944), and were intercrossed (Chandler *et al.*, 1947). None of these polyploid *V. macrocarpon* have shown commercial value (Roper and Vorsa, 1997); however, recently generated tetraploids using newer hybrid germplasm have potential for increased fruit size (Zeldin and McCown, 2002) and a high propensity to set flower buds on fruiting uprights. This trait could potentially avoid the upright biennial bearing character commonly seen in many cranberry plantings (Roper *et al.*, 1993).

In order to generate sufficient numbers of polyploids, an *in vitro* method is utilized that involves an inverted-stem colchicine treatment. Tips are excised from 5 cm microshoots and, after 3 days, the shoots are bundled and inverted into a shell vial containing 0.1 to 1% colchicine in distilled water. After treatment for 24 h in the dark, the microshoots are rinsed and placed upright on fresh shoot culture medium. Nodes at the top of treated shoots fail to grow and eventually turn brown; the amount of stem affected increases with increasing colchicine concentration. Unaffected buds at the basal area of the stem develop quickly and are discarded. The nodes between the dead stem tissue and the unaffected basal area slowly develop and are subcultured after 1 month. In general, one to three polyploid shoots are recovered per treated microshoot; in some cases, 100% of the treated microshoots yield polyploids. Morphological features, e.g. larger leaves and stems, initially identify polyploids and, when flowering occurs, the flowers are also noticeably enlarged (Zeldin and McCown, 2002). In addition, the fruit is both larger and more rounded in shape (Zeldin and McCown, 2002). The larger pollen size of polyploids is used to confirm polyploidy (Altmann *et al.*, 1994; Johansen and von Bothmer, 1994).

Poor sexual fertility is a significant problem in polyploid cranberry, particularly when self-pollinated (Zeldin and McCown, 2002). One generation of breeding and selection using these lines has shown significant improvement in fertility in greenhouse testing (Zeldin and McCown, 2002). Both first- and second-generation plants have been outplanted to examine fertility under field conditions. Whether these tetraploids will prove to be less hardy than the diploid *V. macrocarpon* (as observed with the natural populations of *V. oxycoccus* (Luby *et al.*, 1990)) and with the earlier tetraploid *V. macrocarpon* plants (Galletta, 1975) will be important to determine.

4.1.3. Embryo rescue

Cranberry seeds are relatively small and are dormant at harvest. Their germination usually benefits from a cold stratification period

or heat treatment. Germination is relatively easy; however, young seedlings are intolerant of desiccation and susceptible to root rots and damping-off. In addition, seedlings generated from inbred crosses are often weak and show poor germination. Partially fertile crosses that generate few seeds per fruit will often fail because of fruit abortion. For these and other reasons, embryo rescue is a useful tool for the breeder and accelerates the breeding cycle. The elimination of seed dormancy and the isolation of the seedling in sterile culture so that it can be micropropagated both facilitate rapid germplasm evaluation.

Cranberry seed can be extracted as early as 8 weeks after pollination. The embryos have reached full size by this time, although the seeds have not fully matured and the seed coats have not turned brown. Two approaches can be used to recover viable plants: embryo excision and whole seed germination (E.L. Zeldin and B.H. McCown, unpublished results). With embryo excision, embryos are removed from the seed by slicing off the radicle end of the seed and gently squeezing out the embryo. The embryos are placed on a hormone-free medium since the embryos, as well as newly germinated seedlings, are sensitive to even low levels of cytokinin, which can cause callus and adventitious bud formation on the hypocotyls. Alternatively, seeds are germinated *in vitro* on a hormone-free medium for at least 1 week. After the seed coat has turned brown, the cultures are put into unsealed plastic bags and placed in the dark at 4°C for 12 weeks to break dormancy. Two weeks after the seeds are returned to warm conditions (26°C under continuous light), most of the viable seeds show radicle emergence and, after 4 weeks, up to 100% of the seeds have germinated. After several subcultures, the shoots are ready to remove from microculture.

4.2. Genetic transformation

4.2.1. Breeding objectives

The initial studies involving genetic transformation of cranberry have involved enhancing resistance to certain key insect pests and engineering herbicide resistance.

4.2.2. Protocol

Cranberry was first genetically engineered using particle bombardment of stem sections with a construct that contained *Gus*, *NptII* and *Bacillus thuringiensis* *Bt* endotoxin (Serres *et al.*, 1992). The protocol included a number of steps that were critical to successful recovery of transformed shoots: (i) before bombardment, a 14-day pre-treatment of the stem sections to induce centres of adventitious budding was needed to achieve a maximum number of transient *Gus* events; (ii) because the regenerating tissues grew slowly under kanamycin selection, a long-term selection (7–26 weeks) regime was required. Kanamycin was replenished periodically as a thin liquid overlay to maintain effective and constant selection. Using this protocol, no escapes (non-transformed shoots) were detected among the recovered shoots.

Strategies for the transformation of cranberry using *Agrobacterium*-based methods have been reviewed by Polashock and Vorsa (2003). Recently, a transformation system using *Agrobacterium* has been tested using leaf segments (J.J. Polashock and N. Vorsa, 2001, personal communication). Using the hypervirulent strain EHA105, pCAMBIA vectors (Roberts *et al.*, 1997) and selection with kanamycin, transient *Gus* expression was observed; however, few stable transformants were obtained, even after exhaustive trials. Modifications to the method (sonication, acetosyringone, vacuum infiltration and cultivar selection) made little difference in efficiency of either transient or stable transformation.

4.2.3. Accomplishments

Insect pest resistance. Using a construct that contained *Gus*, *NptII* and *Bt* and particle bombardment, 90% of the recovered transformed and cloned shoots (transclones) contained all three introduced genes (Serres *et al.*, 1992). All transclones possessed the *NptII* gene, verifying the intensity of the kanamycin selection system used. Three-quarters of the transclones expressed *Gus*; however, no consistent effect of the *Bt* gene on insect feeding was detected in feeding

bioassays with the important lepidopteran cranberry pest, blackheaded fireworm (*Rhopobota naevana*) (R. Serres, 1993, personal communication). Few transclones showed intense and uniform *Gus* staining using histochemical assays, but instead, small and seemingly random areas of blue stain were observed with most of the recovered transformants (Serres *et al.*, 1992, 1997). Further study using fluorogenic assays showed that components of the cranberry tissue, possibly flavonols and proanthocyanins, may have been responsible for much of the non-uniform staining by inactivating the β -glucuronidase enzyme produced by the inserted *Gus* gene (Serres *et al.*, 1997). The use of polyvinylpyrrolidone (PVPP) to remove components that inactivated the β -glucuronidase enzyme during extraction resulted in much higher levels of β -glucuronidase being fluorogenically detected (Fig. 8.2.3) as well as a more consistent pattern of *Gus* expression between assays and between transformants. Eliminating this interference by phenolic compounds revealed that *Gus* expression varied greatly among the transclones and within a transclone and depended on the developmental and physiological state of the tissue and the growth environment. In general, younger tissues, shoot tips and microcultured sources all showed the higher detectable β -glucuronidase activities.

R.A. Serres, as reported in Polashock and Vorsa (2003), showed that endogenous compounds that are present in cranberry leaves inhibited the activity of *Bt* endotoxin and therefore could be responsible in part for the lack of *Bt* gene efficacy in the cranberry transclones. When an extract from cranberry leaves was mixed with a commercial *B. thuringiensis* preparation and fed to blackheaded fireworm (*R. naevana*), using microdroplet bioassays, the efficacy of the *B. thuringiensis* preparation was reduced 200% (Polashock and Vorsa, 2003). Likewise, the growth inhibiting effects of a *B. thuringiensis* preparation on a standard test insect, the soybean looper (*Pseudoplusia includens*), was reduced by mixing the *B. thuringiensis* preparation with a cranberry leaf extract (Fig. 8.2.4). Similar effects of phenolic compounds

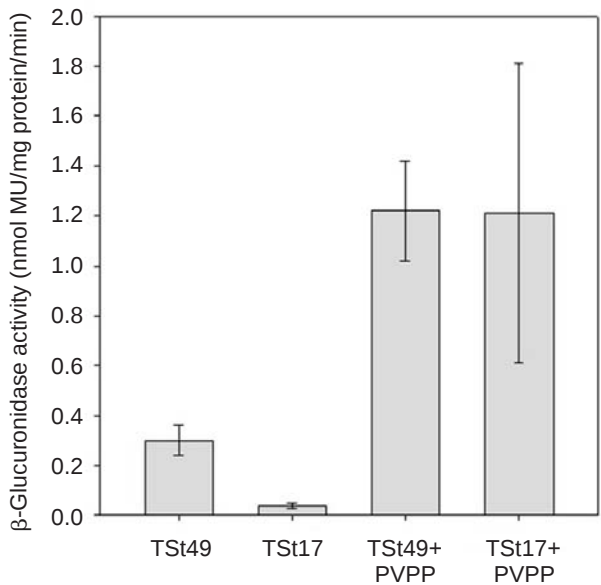


Fig. 8.2.3. Effect of the addition of insoluble polyvinylpyrrolidone (PVPP) (0.04 g/g fresh weight of shoot) during the preparation of aqueous leaf extracts on the detectable β -glucuronidase enzyme activity in two cranberry transclones, TSt49 and TSt17. Shoots were taken from shoot cultures and enzyme activity was determined by fluorogenic assay. Data are mean $\pm$ standard error of three samples.

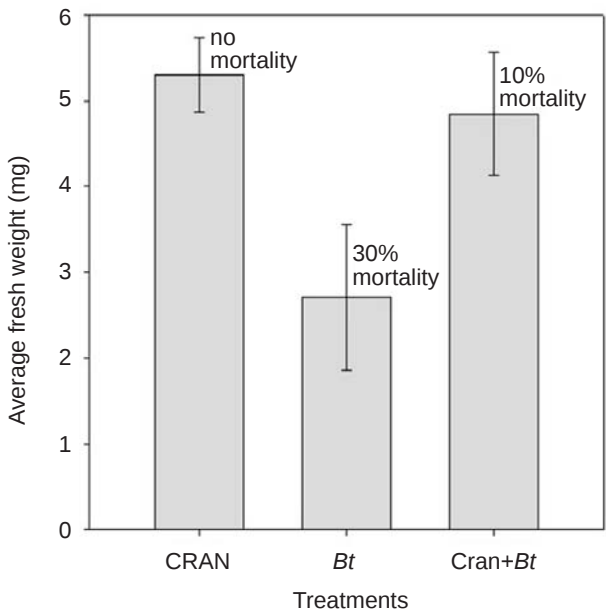


Fig. 8.2.4. Effect of *Bt* σ -endotoxin and cranberry leaf extract on the growth (fresh weight of surviving larvae) and survival (% mortality) of blackheaded fireworm (*Rhopobota naevana*), a common cranberry pest. Larvae were allowed to drink for 1 h microdroplets containing the endotoxin (*Bt*, a lysate from commercial Mycogen's MVP-Bt) and/or an aqueous extract from fresh cranberry leaves (CRAN), after which the larvae fed upon cranberry shoots for 6 days. Mean $\pm$ standard error of ten larvae.

on the efficacy of *B. thuringiensis* have been reported (Luthy *et al.*, 1985; Lord and Undeen, 1990; Navon *et al.*, 1993). As caterpillars consume leaves of a cranberry transclone, the cranberry leaf containing the *Bt* endotoxin is macerated and the cytoplasmic *Bt* is exposed to phenolic vacuolar contents, thereby inactivating the *Bt* endotoxin. A similar mechanism might explain why *B. thuringiensis* sprays for lepidopteran leaf and fruit pest control in cranberry beds have shown inconsistent efficacy.

Herbicide tolerance. To determine if *V. macrocarpon* could be successfully genetically engineered for herbicide tolerance, a study was conducted using the *Bar* gene, which codes for tolerance of the herbicide L-phosphinothricin. L-Phosphinothricin is a naturally occurring amino acid that is a potent inhibitor of all forms of glutamine synthetase occurring in plants (Leason *et al.*, 1982; Wild and Manderscheid, 1984). Inhibition of glutamine synthetase results in the toxic accumulation of ammonia produced by direct uptake, photorespiration, nitrate reduction or protein/amino acid metabolism (Wallsgrave *et al.*, 1987). The *Bar* gene was derived from a common soil bacterium, *Streptomyces hygroscopicus*, and encodes an enzyme that acetylates phosphinothricin, thus inactivating the herbicide (Thompson *et al.*, 1987). The construct had a pUC19 plasmid backbone (Yanisch-Perron *et al.*, 1985) and contained the *bar* gene, driven by the cauliflower mosaic virus (CaMV) 35S promoter (Nagy *et al.*, 1985; Odell *et al.*, 1985), containing the lucerne mosaic virus non-coding leader sequence (Gehrke *et al.*, 1983) 5' to the gene coding sequence and a soybean small subunit gene polyadenylation signal region (Berry-Lowe *et al.*, 1982). The plasmid also contained the *AphII* gene conferring tolerance of the antibiotic kanamycin, which was driven by the nopaline synthetase promoter (McCown *et al.*, 1991).

Attempts to use phosphinothricin as the sole selective agent were unsuccessful. Therefore, the kanamycin selection system (Serres *et al.*, 1992) was utilized; however, selected shoots were further screened in microculture by exposing them to a

bialophos (the dialanine conjugate of phosphinothricin) inhibition assay, using shoot tips from actively growing shoot cultures of putative transformants and placing them on bialophos-amended media. Even at this stage, there was little indication of high levels of herbicide tolerance using the *in vitro* assays. Non-transformed shoots were not killed after 5 weeks' exposure to levels of up to 20 mg/l bialophos in the medium; however, rapid shoot growth (elongation) and the development of adventitious roots were strongly inhibited at levels of 0.1 mg/l bialophos and above. The best regenerated plantlets developed roots on medium containing low levels of bialophos (0.25 mg/l), a concentration that inhibited control shoots (Fig. 8.2.5).

Plants of the best performing selected transgenic plants were then grown outdoors in cold frames and sprayed with increasing herbicide levels to determine if tolerance was efficacious at the whole plant level. Levels as low as 100 ppm glufosinate killed the vast

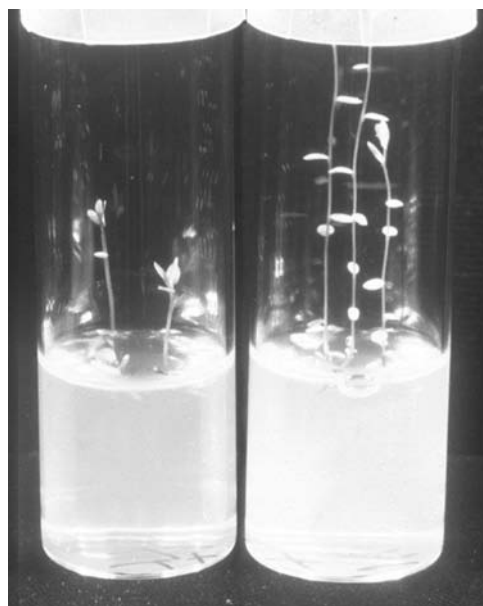


Fig. 8.2.5. Microshoots of *Vaccinium macrocarpon* 'Pilgrim' growing in microculture on a medium amended with 0.25 mg/l bialophos herbicide. Left vial, original untransformed clone; right vial, a transclone containing the *Bar* gene.

majority of non-transformed cranberry shoot tips within 2 weeks; whole plant mortality at 4 weeks escalated with increasing herbicide concentrations up to 300 ppm, where none of the plants survived. Conversely, no plants of the transclone were killed by any herbicide concentration tested and shoot tip survival was moderately impacted only at concentrations > 400 ppm. The plants engineered for herbicide tolerance were then used in a breeding experiment to determine if the trait was transmitted sexually in a stable manner. Not only was the herbicide tolerance transferred to both selfed and cross-pollinated seedlings, but some of the progeny displayed greater herbicide tolerance than the original transclone (Fig. 8.2.6). The full explanation for this effect is not known; however, preliminary Southern blot data indicate that gene dosage or construct rearrangement does not appear to be involved (Zeldin *et al.*, 2002).

It is clear that cranberry selections can be readily transformed with genes of commercial value and, at least for herbicide tolerance, plants of potential commercial importance can be recovered; however, even if such genetically engineered plants prove to be commercially useful in full-scale field

trials, other major limitations to the utilization of such germplasm will probably delay or even fully prevent the release to growers. A major concern is the probability that the transgenes in commercial plantings will 'escape' and be transferred to native cranberry populations as well as to non-genetically engineered commercial plantings of cranberries growing in the same locality. Cranberry is insect pollinated and cultivars and native plants appear to be highly cross-fertile. Therefore, the likelihood of pollen transfer of introduced genes is very high. Although the impact of a herbicide tolerance gene on native ecosystems is probably low and thus tolerable, the uncontrolled 'contamination' of other commercial fields is not acceptable. One approach to minimize such an impact would be an adequate buffer zone between fields of genetically engineered and non-engineered plants. In addition, the genetically engineered lines could be introduced only as tetraploids (Zeldin and McCown, 2003), thereby limiting fruit set or rendering sterile most cross-pollinated seedling progeny and thereby minimizing the potential of spread of the inserted genes via hybrid seedlings.

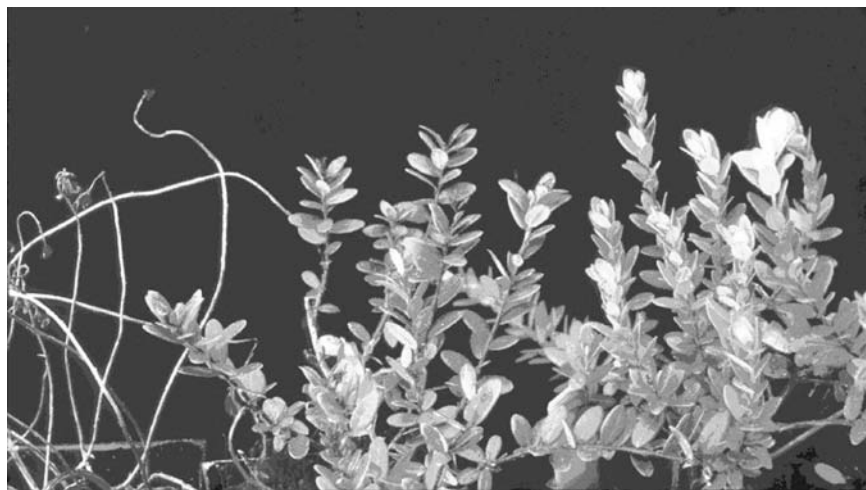


Fig. 8.2.6. Three plants of cranberry (*Vaccinium macrocarpon*) after foliar treatment with 8000 ppm Liberty™ herbicide. Left, untransformed control of 'Pilgrim'; middle, original transclone of 'Pilgrim' engineered with the *Bar* gene; right, hybrid of original transclone and 'HyRed', a new introduction. Although the original transclone did not succumb as did the control, shoot tips were injured. However, the hybrid showed no injury except a temporary delay in growth.

4.3. *In vitro* storage

Germplasm collections exist as field plots in all major cranberry-growing areas, either in experimental stations or on commercial beds. While this has preserved many of the native selections, the germplasm is exposed to risks (contamination by other genotypes, damage by severe weather or disease) and requires considerably more maintenance than normal cranberry beds. The National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon, USA, maintains its collection in individual trays in a greenhouse. This removes much of the risk and allows a number of individuals to be kept in a relatively small area. For long-term storage, the NCGR uses cold storage of micropropagated shoots in sealed bags, which can be kept for months at refrigerator temperatures (Barbara Reed, 2001, personal communication).

5. Conclusions

Microculture can be a critical part of a breeding programme for a woody perennial such as cranberry. As with all woody perennials, a major obstacle to breeding is the time factor, especially the time from seed to seed. Although many of the tasks to which microculture is applied can be done by non-biotechnological methods, employing an array of microculture methods can result in both an increase in efficiency of recovering hybrid progeny and a significant reduction in the time needed to go from seed to seed. Thus, by combining *in vitro* germination, short-term germplasm storage as shoot cultures, micropropagation with accelerated plant growth and crossing under glasshouse conditions, cranberry can be bred reliably on a 2-year cycle. If more extreme measures such as the use of growth retardants to accelerate bud set are employed, the generation time can be further reduced (Serres and McCown, 1994).

Modifying the cranberry genome by use of genetic engineering is feasible. It is not clear if genetic engineering is needed in a breeding programme for cranberry since the species has a high amount of inherent and

unexplored genetic diversity. A significant problem is determining what traits are actually present in this relatively unexplored germplasm base. The genomic tools of biotechnology (restriction fragment length polymorphisms (RFLPs), SCARs, marker-assisted selection) may prove useful in deciphering cranberry native germplasm.

Some traits appear to be so readily improved by plant breeding that genetic engineering technologies have little application. For example, cranberries are grown and marketed primarily for juice, and extractable flavonoid pigments, especially anthocyanins, are a premium. The anthocyanin pigment profile of *V. macrocarpon* is relatively simple, consisting of six major anthocyanins (Mazza and Miniati, 1993). Since many of the critical genes and genetic controls involved in the anthocyanin synthetic pathways are becoming elucidated (Wienand *et al.*, 1990; Gong *et al.*, 1997; Chawla *et al.*, 1999; Gandikota *et al.*, 2001), modifying pigment content through genetic engineering might seem to be a reasonable goal. A recent and limited breeding effort has demonstrated rapid progress in markedly increasing fruit total pigment content as well as the timing of pigment accumulation (McCown and Zeldin, 2003). Although total pigment quantity can be readily enhanced using inter-cultivar crossing and selection, the profiles of specific pigments making up the pigment content remain remarkably stable between cultivars (Mazza and Miniati, 1993). There is therefore an opportunity to use genetic engineering for modifying the pigment quality.

Cranberry products are considered by consumers to be both healthful and natural, whereas genetic engineering is considered by many consumer groups to be unnatural (Gaskell *et al.*, 2000). As long as this difference in perceptions exists, genetic engineering will be difficult to employ for cranberry crop improvement. Indeed, the further commercial development of herbicide-tolerant cranberry has been sidelined due to such concerns.

Future breeding objectives of cranberry will include more emphasis on fruit quality, increased efficiency and lowered impact of production. Two goals seem to be particu-

larly open for future exploitation. Modifying or changing the flavour of cranberry fruit is an obvious potential goal. For example, inserting a gene that codes for a natural sweetener protein such as brazzein (Assadi-Porter *et al.*, 2000) could lead to a new array of products using the otherwise overly tart and non-sweet cranberry. In addition, improving the health benefits of cranberry fruit and processed products will probably emerge as a major genetic improvement goal. However, aggressively tackling the health aspects of cranberries must await the identification of those physiochemical factors present in the fruit that are reliably beneficial. Such work has already begun; Polashock and Vorsa (2003) directed an effort to clone four structural genes and one putative regulatory gene in the cranberry flavonoid biosynthetic pathway. The genes were isolated by screening a genomic DNA library with a heterologous probe or by polymerase chain reaction (PCR) using degenerate primers based on

published sequences. A cranberry dihydroflavonol reductase has been tested in a model tobacco transformant system, but not in cranberry (Polashock *et al.*, 2002). Whether genetic engineering will play a unique and important role in achieving the goal of modifying fruit quality is an open question.

Finally, cranberry possesses all the complexities of any woody fruit crop – singulated fruiting, spur-like shoots, preformed buds, biennial bearing fruiting units, fruit number/fruit size tradeoffs. However, mature cranberry plants are small and readily grown, the cranberry genome size is not huge and estimated to be 1.2 pg (Costich *et al.*, 1993) and, for a woody plant, cranberry is easy to manipulate using biotechnological methods. These traits make cranberry an intriguing choice as a biotechnological test organism to explore new concepts before tackling more cumbersome fruit crops (Serres *et al.*, 1994b).

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9

Fagaceae

The archaic *Fagaceae* Dumortier (syn. *Quercaceae* Martinov, *Castaneaceae* Adams), the beech family, belongs to the order of cup-bearing woody angiosperms, *Fagales*, of the subclass *Hamamelidae* (*Amentiferae*). The *Fagaceae* is by far the largest family in the order, with nine genera and approx. 900 species of trees and shrubs, which are widely distributed throughout temperate and subtropical climatic zones, mostly in the northern hemisphere, with the greatest species diversity found in East and South-east Asia and North America (Watson and Dallwitz, 1992 onwards).

The *Fagaceae* was formerly known as the family *Cupuliferae* because an emblematic botanical feature of its members is the cupule (or hull), a complex cup-like structure that subtends and partially or completely covers the fruits. The cupule has been interpreted as a condensed, partial inflorescence formed by fusion of stem axes with several orders of branching, bearing bracts that are modified as scales and/or spines (Fey and Endress, 1983); however, its anatomical origin is still debated.

Two subfamilies are recognized within the *Fagaceae*: (i) *Fagoideae*, containing the genera *Fagus* (beeches), *Quercus* (oaks), *Colombobalanus*, *Formanodendron* and *Trigonobalanus*; and (ii) *Castaneoideae*, containing *Castanea* (chestnuts and deciduous chinkapins), *Castanopsis* (evergreen chinkapins), *Chrysolepis* and *Lithocarpus* (tanoak). These genera make up a significant part of the broad-leaved forests of northern temperate regions, providing habitat and food sources for local wildlife populations. From an economic viewpoint, *Fagus*, *Quercus* and *Castanea* are the most important genera in the family. Beeches, chestnuts and oaks are sources of highly valuable timber and nuts. Moreover, they are among the estimated 3% of all plant species that form ectomycorrhizae, including two of the most valuable of edible mushrooms, *Boletus edulis* Bull. and *Amanita caesarea* Schw. (Álvarez, 1984). Beechnuts and acorns of several white oak species have been used by people for food, livestock feed and other purposes for thousands of years (Reighard, 1989a,b), and the nuts of *Lithocarpus* and *Castanopsis* are a food source in some countries of South-east Asia.

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9.1 *Castanea* spp. Chestnut

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1. Introduction

1.1. Botany and history

Chestnut trees were designated as *Fagus castanea* L. until 1754, when Miller proposed the genus *Castanea*, which comprises up to 13 species of chestnut trees and chinkapins native to temperate Asia, North America and Europe, with greatest diversity in South-east Asia and eastern USA (Camus, 1929; Johnson, 1988). Chestnuts probably originated in Asia Minor (Camus, 1929) and China (Chunji *et al.*, 1993; Liu, 1993). Pollen and fossil evidence from two ancient species, *C. ungeri* Herr and *C. kubiiji*, indicates that chestnuts are at least 40 million years old and were previously dispersed throughout a wider area than their current range (Camus, 1929). The precise number of species is uncertain due to the widespread use of synonyms and the lack of accurate characterization for some species of chinkapins. In addition to the four most important species, *C. crenata* Siebold & Zucc. (Japanese chestnut), *C. dentata* (Marshall) Borkh. (American chestnut), *C. mollissima* Blume (Chinese chestnut) and *C. sativa* Miller (European chestnut), other species also produce edible nuts that have potential (Table 9.1.1).

Chestnuts are deciduous, with alternate, simple leaves 20–30 cm long and 3–6 cm

wide, oblong to lanceolate, with a serrate margin and an acute to acuminate apex. They are shade-tolerant, fast-growing trees that require a warm, moderately humid climate and prefer deep, well drained, slightly acidic (pH 4.5–6.5) soil, although they can thrive on a wide variety of sites and soils. They are found in small groves, in mixed broadleaved forests or as nut orchards. Chestnuts are long-lived trees, with a life expectancy of 300–1000 years. They exhibit a wide variety of forms, from shrubs to canopy trees. Under good growing conditions, the European chestnut can attain 30 m height, > 2 m in trunk diameter and a crown width of 23–35 m. Some specimens of American chestnut are reported to have been even larger.

The flowering habit of *Castanea* is monoecious, with male and female flowers grouped in either unisexual or bisexual inflorescences, which are catkins (or aments), borne on the current season's shoots. Staminate flowers are borne in long (15–30 cm), slender, unisexual, catkins located on the lower portion of the shoot or on the distal part of the bisexual catkins near the terminal portion of the shoot. Pistillate inflorescences usually consist of three female flowers clustered in a prickly involucre at the base of the bisexual catkins. Each female flower has six to nine styles and contains six to 16 anatropous ovules found in the upper part of the axis of an inferior six-

Table 9.1.1. Prominent *Castanea* species, their geographic distributions and some useful characteristics.

Species/Section	Distribution	Common name(s)	Characteristics	Nuts
<i>C. crenata</i> Siebold and Zuccarini <i>Castanea</i>	Native to Japan – Hokkaido (SW), Honshu, Kyushu, Shikoku Naturalized in South Korea and China	Japanese chestnut	Small, apple-shaped tree (< 15 m), early branching Precocious bearing, high yield, early ripening Resistant to ink disease Moderate resistance to blight Susceptible to gall wasp	Three large nuts per bur, relatively poor quality Fibrous pellicle
<i>C. detata</i> (Marshall) Borh <i>Castanea</i>	Eastern and south-eastern USA	American chestnut	Large, fast-growing tree before blight (< 35 m), Surviving as an understorey shrub The most blight-susceptible species Susceptible to ink disease	Three small nuts per bur, very sweet
<i>C. henryi</i> (Skan) Redher et Wilson <i>Hypocastanon</i>	Middle China from the coast to the far temperate west	Zhui Li Henry chinkapin Chinese timber chinkapin Pearl chestnut Willow-leaved chestnut Chinese chestnut	Large tree (≤ 35 m) Mainly used for its good timber High resistance to blight Variable resistance to ink disease Susceptible to gall wasp	One or rarely two per bur, very small cone-shaped nuts
<i>C. mollissima</i> Blume <i>Castanea</i>	From south China to north of Beijing Naturalized in South Korea and Vietnam	Chinese chestnut	Small spreading tree (≤ 10 m), early branching The most blight-resistant species Resistant to ink disease	Three nuts per bur, sweet
<i>C. pumila</i> Miller <i>Balanocastanon</i>	Native to North America From New Jersey and Pennsylvania south to Florida and Texas	Allegheny chinkapin	Highly variable growth habit, from a creeping sub-shrub to small tree Precocious bearing Susceptible to blight and ink disease	One small nut per bur, very sweet
<i>C. sativa</i> Miller <i>Castanea</i>	Native to southern Europe and Asia Minor North Africa: Algeria, Morocco, Tunisia Widely cultivated and naturalized elsewhere in Eurasia	European chestnut, Spanish chestnut Sweet chestnut	Large tree (≤ 30 m), variable growth habit Good timber Strong branches Susceptible to ink disease, variable susceptibility to blight Small tree or shrub (≤ 10 m)	Three large nuts per bur, good quality
<i>C. seguinii</i> Dode <i>Castanea</i>	East and central China	Mao li Chinese dwarf chestnut Seguin chestnut	Dwarfing rootstock Highly variable flowering habits, very precocious bearing Blight-resistant Source of blight resistance genes different from <i>C. mollissima</i>	One very small nut per bur Used for flour

to nine-celled ovary (Elorrieta, 1949; Paglietta and Bounous, 1979). The mature ovule consists of two integuments and a long narrow nucellus with a small embryo sac located at the micropylar end (Botta *et al.*, 1995).

Chestnuts are annual bearers and begin to bear earlier than most fruit and nut trees. Depending on the species, one to three chestnut fruits are enclosed in a spiny bur and vary in colour from light brown to dark red-brown to black and in weight from 5 to 35 g. They ripen 75 to 110–120 days after pollination, and most fall free from the burs, facilitating harvesting. Each nut may contain one or more embryos.

1.2. Importance

The chestnut is a tree with great social value and has been identified with the cultures, economies and religions of different peoples from many countries. Chestnuts have traditionally been cultivated in China, Korea, Japan and the Mediterranean region. They were under cultivation 6000 years ago in China. In Europe, ancient Greeks were among the first to cultivate the nut, at least 3000 years ago, and introduced the European chestnut from Asia Minor to southern Europe and North Africa. The exceptional nutritional value of chestnuts has long been recognized; ancient Greeks and pre-Roman tribes venerated the chestnut tree and considered chestnuts to be superior to almonds, hazelnuts and walnuts. By the 1570s, de Montaigne wrote in his *Journal du Voyage* that Roman legions in the Gallic War subsisted on chestnuts, which were considered a 'bread of the forest', and they called the chestnut tree *arbus panis*.

Currently, chestnuts constitute the only commercially important nut crop within the family. There are chestnut-based industries in most chestnut-producing countries, and even many countries far from the chestnut range have developed industries for processing. Chestnuts are one of the most significant nut crops in the temperate zone. Despite increasing demand, world production of chestnuts has declined progressively over the last century, mainly due to fungal diseases and insect

pests, which not only have devastated populations of chestnut trees throughout their range, but also have limited the establishment of new chestnut-growing areas. Current chestnut production in Europe is approx. two-thirds of the level reported in the 1950s.

Worldwide, average chestnut exports for 1990–1998 are estimated to be 0.1 Mt annually (FAOSTAT, 2004). This is far less than the total chestnut production (1.04 million t), which is very difficult to estimate accurately because much of the chestnut crop is consumed locally while still fresh. The leading chestnut-producing countries are China, Turkey, Korea, Italy, Japan, Spain, Portugal, France and Greece, while the leading markets are Japan, which imports more than three times its own production, France, Hong Kong, Switzerland, Brazil, the USA, Germany, Austria and Italy.

Nutritionally, the chestnut is similar to wheat and rice and considered 'a grain that grows on a tree' (Burnett, 1987). Biochemically, the composition of chestnuts is very different from that of any other major nut (Charro and Barreiro, 1957; Scharz, 1990; Payne *et al.*, 1993; Viéitez *et al.*, 1996), containing approx. 170 calories/100 g and a high carbohydrate content (60–70% dry weight (DW)). The high-quality protein (10–15%) is characterized by an amino acid balance comparable to that of fresh eggs, but without cholesterol. Its fat content (1–4%) is the lowest of all the major edible nuts.

Chestnuts can be eaten raw, roasted as snacks, boiled, dried or cooked following many well-known popular recipes, and served as vegetables, soups or purées or candied as marrons glacés. Dried chestnuts are milled into highly prized flour for pastries (Bergougnoux *et al.*, 1978; Burnett and Wallace, 1987).

There is great interest in restoring chestnut culture and extending its cultivation. World demand for chestnuts is increasing at a much higher rate than production, exceeding the demand for almonds and walnuts combined, and international chestnut prices have steadily increased. Chestnut trees are well suited for low input agroforestry systems and offer advantages as a profitable multiple use crop for producing nuts and timber.

1.3. Breeding and genetics

Chestnuts are self-sterile (Stout, 1926) and usually protandrous. They are predominantly wind-pollinated, but small thrips and bees also play a role in cross-pollination (Gallastegui, 1926). Generally, anthesis lasts for 20–30 days. The male catkins bloom first and shed pollen approx. 5 days before the stigmas are receptive. The period of maximum receptivity lasts for 3–4 weeks (Bergougnoux *et al.*, 1978). The floral phenology has been described by Bounous *et al.* (1992), and the floral biology in relation to phenological stages from anthesis to fruit set has been reviewed by Botta *et al.* (1995).

Castanea displays high levels of genetic variability within and among species as a result of their wide geographical ranges, out-crossing habit and long history of cultivation. This genetic variability is evident in the great number of local genotypes and cultivars that have been developed by selection of the best seedling progenies and grafts. A study of genetic variation among 12 populations of *C. dentata* using allozymes showed that about 10% of the allozyme diversity resided among populations (Huang *et al.*, 1998), which is similar to the among-population diversity reported for *C. sativa* by Pigliucci *et al.* (1990). *Castanea* species are diploid, with $2n = 2x = 24$ (Dermen and Diller, 1962; Jaynes, 1962; Li, 1981), and the different species hybridize readily (Elorrieta, 1949; Jaynes, 1964).

Chestnut breeding has involved traditional strategies, e.g. selection within seedling populations from higher yielding orchards or from selected elite trees. Another option for genetic improvement is clonal selection and vegetative propagation of superior genotypes. Clonal selection is the best option for the genetic improvement of heterozygous species such as chestnut. In fact, most cultivars have originated from scion selections grafted on to seedling rootstocks.

1.3.1. Breeding objectives

Chestnut breeding objectives have varied according to the final use of the nuts and the specific needs of individual countries. In

general, breeding programmes have addressed: (i) improvement of nut production, e.g. nut quality (size, flavour, sweetness and ease of peeling), growth habit, earlier and higher production; and (ii) resistance to diseases and pests.

Chestnut trees in Europe and the USA have experienced massive dieback caused by two fungal diseases: (i) ink disease, also known as *Phytophthora* root rot; and (ii) chestnut blight canker. Ink disease is caused by *Phytophthora cambivora* (Petri) Buism. (formerly *Blepharospora cambivora* (Petri)). Discovered first in Portugal in the 1830s (Taveira Fernandes, 1966), ink disease has killed thousands of European chestnuts. In the eastern USA, ink disease, caused by *Phytophthora cinnamomi* Rands, also affected the American chestnut and destroyed entire populations of some chinkapin species (Milburn and Gravatt, 1932; Gravatt and Crandall, 1945).

Chestnut blight canker, caused by *Cryphonectria parasitica* (Murr.) Barr (formerly *Endothia parasitica* (Murr) Anderson), caused the death of millions of American chestnuts over an area of 3.6 million ha during the early 20th century. This epidemic was a major ecological disaster and nearly exterminated the American chestnut as a species. Currently, the tree exists as a minor component of eastern hardwood forests, surviving as sprouts from old stumps and root systems, since the blight only kills the tissues above the canker, and does not penetrate the roots (Anagnostakis, 1995). Commercial chestnut orchards in the eastern USA are entirely based on Chinese and Japanese chestnuts, while, on the Pacific coast, Asian and European chestnuts are cultivated. *C. sativa*, in general, is less susceptible to blight than the American species, but many European chestnuts have also been killed.

Many methods have been used to eradicate or to control these diseases, e.g. quarantine, mechanical methods and chemical control. None of these approaches have been effective in orchards or forests. In Europe, trees have been protected from ink disease by removing soil around the root collar, allowing air drying of the upper roots and/or application of copper carbonate around the root collar. Blight control has involved identification of trees

with natural disease resistance to use as breeding stock; however, progress has been very slow. Natural remission of chestnut blight in Italy (Biraghi, 1953) is due to the natural occurrence of debilitated strains of *C. parasitica* (Grente and Sauret, 1969) that are infected by a cytoplasmically replicating double-stranded RNA (dsRNA) hypovirus (MacDonald and Fulbright, 1991). Hypovirulent strains of the fungus may become the basis for biological control of the disease (Anagnostakis, 1977, 1982; Griffin, 1986; MacDonald and Fulbright, 1991). Previously, transmission of the viruses to other fungal strains via anastomosis was restricted to vegetatively compatible strains of *C. parasitica* (Anagnostakis, 1977, 1982). Nuss and colleagues (Choi and Nuss, 1992; Chen *et al.*, 1993; Nuss, 1993) demonstrated that hypovirulence could be engineered into the fungus using a cDNA copy of the viral genome, thereby allowing transmission of the virus to vegetatively incompatible strains via mating.

In Asia, *C. mollissima* and *C. crenata* are resistant to ink disease and chestnut blight, although the levels of resistance vary; however, many chestnut orchards and wild populations of these and other *Castanea* species have been destroyed by repeated infestations of the chestnut gall wasp (*Dryocosmus kuriphilus* Yasumatsu), which is endemic to China and naturalized in Korea and Japan. The insect was also introduced accidentally into the USA and has caused significant losses (Payne *et al.*, 1983). There are no other organisms that threaten the chestnut (MacDonald, 1993).

Successful establishment of new chestnut plantings is limited by the lack of improved genotypes. Several barriers limit the genetic improvement of the genus and the extension of chestnut culture, including lack of superior genotypes due to the difficulty of vegetative propagation, high heterozygosity, large size, long juvenile period and lack of markers for early progeny selection.

1.3.2. Breeding accomplishments

The history of chestnut breeding has been described by MacDonald *et al.* (1978), Smith and MacDonald (1982), Burnett and Wallace

(1987), Double and MacDonald (1992), Antognozzi (1993) and Saleses (1999). Methods for pollen collection and storage and for hand pollination have been described (Gallastegui, 1926; Maynard, 1991a; Rutter, 1991); however, problems exist with regard to selecting the best parents from the large available germplasm resources and introgressing into these parents the resistance genes from various Asian species.

The decimation of chestnut trees by ink disease and chestnut blight in Europe and America stimulated hybridization among chestnut species. Breeding chestnuts for improved nut quality was initiated in America by Van Fleet (1914), who obtained the first four hybrids in 1903: *C. pumila* × *C. sativa*, *C. pumila* × *C. dentata*, *C. sativa* × *C. dentata* and *C. pumila* × *C. crenata*. Couderc (1919) in France, with the objective of studying ink disease, and Gallastegui (1926) in Spain obtained the following interspecific hybrids: *C. crenata* × *C. sativa*, *C. sativa* × *C. crenata* and *C. sativa* × *C. mollissima*. These hybridization programmes progressed very slowly, because of difficulties measuring resistance levels and problems associated with vegetative multiplication of the hybrids. After the International Chestnut Commission was established by the Food and Agriculture Organization (FAO) in 1951 to study and combat both chestnut diseases, various European countries re-established or initiated breeding programmes for ink disease resistance.

In Spain, within the framework of the Plan de Mejora y Regeneración del Castaño, approx. 12,000 ink disease-resistant *C. sativa* × *C. crenata* hybrids were obtained from more than 263,000 chestnut genotypes that had been tested for resistance to *Phytophthora* (Viéitez, 1960). Included in this group were trees with natural field resistance from within native populations devastated by ink disease, which for the most part had hybrid genotypes. F₁ hybrids were obtained through controlled pollinations of *C. sativa* × *C. crenata*, while F₂ and F₃ generations were obtained, respectively, from open pollinations of Gallastegui's (1926) F₁ and F₂ *C. sativa* × *C. crenata* hybrids with *C. crenata*

and *C. mollissima* pollen. The cambium of the main roots was inoculated at the root collar with a homogenized mixture of 19 fungal strains, the virulence of which had been established previously by testing on susceptible chestnuts (Viéitez, 1960). Inoculations were made under edaphic conditions that were highly permissive to the build-up and spread of fungal inocula, and were repeated over 3 successive years. Trees showing high levels of resistance were propagated by mound layering to select those genotypes that appeared to be superior, which were subsequently used as mother trees in stool beds for further selection and commercial production.

Efforts by the US Department of Agriculture (USDA) to breed a blight-resistant hybrid chestnut by crossing Asian species with American chestnut have generally been unsuccessful (Burnham, 1988; Griffin, 2000). Based on Clapper's (1952) hypothesis that blight resistance may be controlled by as few as two genes, Burnham (1981) proposed applying the back-cross method to produce blight-resistant American chestnuts. The American Chestnut Foundation (TACF) implemented the back-cross breeding programme designed by Burnham (1988). Genes for blight resistance from *C. mollissima* are being transferred to *C. dentata* by back-crossing Chinese $\times$ American hybrids ($C \times AF_1$) showing the highest resistance to American chestnut three or more times and then inter-crossing the most blight-resistant progeny to obtain the blight resistance of Chinese chestnut and the forest tree phenotype of American chestnut. The American Chestnut Co-operators Foundation (ACCF) has also distributed seeds and seedlings from seed orchards established with grafts from large, surviving American chestnut trees. Controlled pollinations among surviving genotypes from the same geographic region have yielded high percentages of progeny with low levels of blight resistance (Griffin, 2000).

Currently, there is no effective vegetative propagation method for mature chestnuts. Although chestnuts can be propagated either by grafting or budding, as well as by layer-

ing and rooted cuttings, the success of these approaches has been limited. Vegetative propagation of chestnuts has been described (Schad *et al.*, 1952; Viéitez, 1952, 1974; Keys, 1978; Elkins *et al.*, 1980; Bazzigher *et al.*, 1984; Lagerstedt, 1987; Viéitez *et al.*, 1996). Selected chestnut scions are easily grafted or budded on to seedlings of the same cultivar or on to clonal hybrid rootstocks; however, grafting and budding are time-consuming and there are serious problems with the disease susceptibility of seedling rootstocks and graft incompatibility or delayed failure of the graft union, especially with *C. mollissima* rootstocks (McKay, 1947; McKay and Jaynes 1969; Huang *et al.*, 1994b). Production of self-rooted trees by layering-derived shoots and stem cuttings is hindered by the fact that chestnuts are particularly difficult to root.

Stool bed layering is mostly used for commercial production of European and Japanese chestnut varieties and ink disease-resistant hybrids. Layering is a lengthy procedure and requires large numbers of mother plants and space. Girdling the lower portion of shoots and/or application of auxin is required to induce rooting. Moreover, there is strong clonal variation in rooting responses (Schad *et al.*, 1952; Viéitez, 1952, 1953, 1955; Solignat, 1964).

The rooting of stem cuttings is potentially the most cost-effective method for mass production of selected chestnut clones; however, rooting of chestnut cuttings has been generally unsuccessful, and has been inconsistent over succeeding years. The rooting capacity of chestnut cuttings appears to be closely related to juvenility (Clapper, 1952; Urquijo, 1952; Viéitez, 1952, 1956, 1963; Graves and Nienstaedt, 1953; Pease, 1953; Jaynes, 1961; Solignat, 1964; Viéitez and Viéitez, 1976; Gesto *et al.*, 1981; Vázquez and Gesto, 1982; Viéitez *et al.*, 1987; Rinallo and Mariotti, 1993a,b). Although some success has been obtained with the use of etiolation as a mother plant pre-treatment, cuttings from adult chestnuts are difficult to root. Anatomical factors and several physiological and biochemical events correlated with poor rooting of chestnut cuttings have been described (Viéitez, E., 1992).

Table 9.1.2. Genetic markers used with *Castanea*.

Species	Marker type/studies	References
<i>Castanea</i>	Morphological markers	McKay (1960)
<i>Castanea</i>	Morphological markers	Jaynes (1963)
<i>C. crenata</i> ; <i>C. mollissima</i> ; <i>C. sativa</i>	Isozymes; varieties	Sawano <i>et al.</i> (1984)
<i>Castanea sativa</i>	Isozymes; varieties	Saiz de Omeñaca <i>et al.</i> (1984)
<i>Castanea</i>	Isozymes; peroxidase	Santamour <i>et al.</i> (1986)
<i>C. sativa</i>	Isozymes; varieties	Fineschi (1988)
<i>C. mollissima</i>	Isozymes; peroxidase; graft compatibility	Santamour (1988)
<i>C. sativa</i>	Isozymes	Fineschi <i>et al.</i> (1990)
<i>C. sativa</i>	Isozymes; characterization	Villani <i>et al.</i> (1991a,b)
<i>Castanea</i> spp.	Isozymes	Anagnostakis (1991)
<i>C. mollissima</i>	Inventory varieties	Liu (1992)
<i>C. dentata</i>	Blight resistance	Bernatzky and Mulcahy (1992)
<i>C. dentata</i>	RFLPs; blight resistance	Ellingboe (1992)
<i>C. sativa</i>	Isozymes; varieties	Conedera <i>et al.</i> (1993)
<i>C. sativa</i> × <i>C. crenata</i>	Polypeptides; ageing	Amo-Marco <i>et al.</i> (1993)
<i>Castanea</i> (Fagaceae)	Varieties; evolution	Frascaria <i>et al.</i> (1993)
<i>C. sativa</i>	Isozymes	Villani <i>et al.</i> (1993)
<i>C. sativa</i>	Isozymes; inventory varieties	Müller-Starck <i>et al.</i> (1993)
<i>C. sativa</i>	Isozymes; varieties	Villani and Cherubini (1994)
<i>C. dentata</i> ; <i>C. mollissima</i> ; <i>C. seguinii</i>	Isozymes; varieties; allozymes	Huang <i>et al.</i> (1994a,c)
<i>Castanea</i>	RAPDs; blight biocontrol	Arisan-Atac (1995)
<i>Castanea</i>	PCR; molecular mapping	Bergamini Mulcahy <i>et al.</i> (1995)
<i>Castanea</i> spp.	Morphological markers	Kotobuki (1996)
<i>Castanea</i> ×	Molecular mapping; blight resistance	Kubisiak <i>et al.</i> (1997)
<i>C. sativa</i>	RAPDs/cultivar typing	Galderisi <i>et al.</i> (1998)
<i>C. dentata</i>	RAPDs; allozymes	Huang <i>et al.</i> (1998)
<i>C. dentata</i>	DNA markers; mutations	Kubisiak (1999)
<i>C. sativa</i> ; <i>C. sativa</i> × <i>C. crenata</i>	Polyphenols; phase change	Fernandez-Lorenzo <i>et al.</i> (1999)
<i>C. sativa</i>	Microsatellite markers; varieties	Botta <i>et al.</i> (1999)
<i>C. henrii</i> ; <i>C. mollissima</i> ; <i>C. seguinii</i>	Allozymes; population structure	Lang and Huang (1999)
<i>C. pumila</i> var. <i>ozarkensis</i> .	RAPDs; isozymes; population structure	Dane <i>et al.</i> (1999)
<i>C. sativa</i> ; <i>C. sativa</i> × <i>C. crenata</i>	RAPDs; varieties; hybrid rootstock identification	Santana <i>et al.</i> (1999)
<i>C. sativa</i>	RFLP; population structures	Fineschi <i>et al.</i> (2000)
<i>C. sativa</i>	Isozymes; RAPDs; ISSRs; linkage mapping	Casasoli <i>et al.</i> (2001)
<i>C. sativa</i>	RAPDs; ISSRs	Goulao <i>et al.</i> (2001)

2. Molecular Genetics

2.1. Molecular markers

Molecular genetics has only recently been applied to *Castanea*, but promises to bring about improvement of the genus, especially with respect to its applications for mapping disease resistance genes. Prior to the development of molecular markers, morphological markers and isozymes were used for species and cultivar identification, breeding, population genetics and studies of genetic diversity (Table 9.1.2). Bernatzky and Mulcahy (1992) discussed the application of restriction fragment length polymorphisms (RFLPs) for marker-assisted selection, as a means of reducing the number of generations of back-crosses required to obtain the desired combination of blight resistance from *C. mollissima* and other phenotypic traits from *C. dentata*. A genetic linkage map consisting of isozyme, RFLP and random amplified polymorphic DNA (RAPD) markers was constructed for chestnut using a three-generation *C. dentata* × *C. mollissima* pedigree (Kubisiak *et al.*, 1997). Two isozymes, 12 RFLPs and 170 RAPDs were mapped to 12 linkage groups. Canker expansion data from inoculations of the F₂ mapping population with *C. parasitica* were compared with marker genotype data to identify genomic regions associated with resistance. Three regions of the genome were found to have a significant effect on host response, explaining up to 42% of the total variation for canker size. A genetic linkage map has also been constructed for *C. sativa*, using the two-way pseudo-test cross strategy. Ninety-six members of an F₁ full-sib family were genotyped with 381 molecular markers, including 311 RAPDs, 65 inter-simple sequence repeats (ISSRs) and five isozymes (Casasoli *et al.*, 2001). Kubisiak (1999) showed that RAPD markers could be used to distinguish among six different *Castanea* species and *C. mollissima* × *C. dentata* F₁ hybrids, and to determine that a tree of unknown origin has *C. dentata* ancestry. He concluded that RAPDs could be used to determine the genetic identity of putative American chestnut trees for potential inclu-

sion in breeding programmes. Galderisi *et al.* (1998) described and RAPD-based procedure for molecular typing of *C. sativa* cultivars, while both RAPD and ISSR markers have been used to characterize variability among *C. sativa* cultivars (Goulao *et al.*, 2001).

2.2. Genomics

Genomics could provide a means for elucidating important functions that are essential for adaptability. Data generated by genomics tools could be applied to predict crop performance in different environments and for genome characterization. The approach could provide useful information for development of new cultivars adapted to a range of distinct environments, thus enhancing crop productivity and helping to extend chestnut cultivation to marginal lands.

3. Micropropagation

Since the first *in vitro* report involving chestnut (Jacquiot, 1947), there have been numerous studies involving *in vitro* culture of the tree (Table 9.1.3). Early studies addressed the physiology of host-pathogen interactions (Grente and Sauret, 1961; Borrod, 1971a,b; Hebard and Kaufman, 1976, 1978), embryo culture (Vázquez and Viéitez, 1962, 1965, 1966) and morphogenesis (Trippi, 1963; Hu and Scrivani, 1977; Keys, 1977, 1978; Viéitez *et al.*, 1978; Park and Hung, 1979; González, 1981; Jeune, 1982). The absence of an efficient mass propagation method for selected genotypes has made micropropagation the primary objective.

Micropropagation involving the proliferation of axillary shoots from juvenile explants and rooting of isolated shoots has been reported for *C. sativa* (Viéitez and Viéitez, 1980a,b, 1982), *C. dentata* (McPheeters *et al.*, 1980; Keys and Cech, 1981, 1982) and *C. mollissima* (McPheeters *et al.*, 1980). These results provided the basis for micropropagation using other juvenile materials of *C. sativa* (Rodríguez, 1982a,b; Chevre *et al.*, 1983; Strullu *et al.*, 1986; Mullins, 1987; Piagnani and Eccher, 1988; Ivanova and Erdelsky,

Table 9.1.3. *In vitro* axillary bud enhancement and micropropagation in *Castanea*.

Species	Explants	Response /studies	Reference
<i>C. sativa</i>	Zygotic embryo axes Nodes from seedlings	Axillary shoot proliferation Plantlets in soil	Viéitez and Viéitez (1980a,b)
<i>C. dentata</i> ; <i>C. mollissima</i> ; <i>C. mollissima</i> × <i>C. dentata</i>	Zygotic embryo axes	Axillary shoot proliferation	McPheeters <i>et al.</i> (1980)
<i>C. dentata</i>	Zygotic embryo axes Axillary buds from seedlings	Axillary shoot proliferation Plantlets in soil	Keys and Cech (1981, 1982)
<i>C. sativa</i>	Axillary buds from mature trees	Axillary shoots and plantlet formation	Biondi <i>et al.</i> (1981)
<i>C. sativa</i>	Meristems Seeds	Axillary shoot proliferation Plantlets in soil	Rodríguez (1982a,b)
<i>C. sativa</i>	Nodes from seedlings	Axillary shoot proliferation Plantlets in soil	Viéitez and Viéitez (1982)
<i>C. sativa</i> × <i>C. crenata</i>	Shoot tips and axillary buds from mature trees	Micropropagation Plantlets in soil	Viéitez <i>et al.</i> (1983)
<i>C. sativa</i>	Shoot cultures	Rooting Anatomical events	Viéitez and Viéitez (1983)
<i>C. sativa</i>	Axillary buds from seedlings and mature trees	Micropropagation	Chevre <i>et al.</i> (1983); Chevre and Salesses (1984, 1985)
<i>C. dentata</i>	Shoot apex from mature trees	Micropropagation Plantlet in soil	Read <i>et al.</i> (1985a,b)
<i>C. sativa</i> × <i>C. crenata</i>	Shoot cultures	Vitrification Anatomical and chemical studies	Viéitez <i>et al.</i> (1985, 1988)
<i>C. mollissima</i>	Apical buds and nodes	Axillary shoot proliferation Rooting	Qiguang <i>et al.</i> (1986)
<i>C. sativa</i>	Axillary buds and shoot tips from seedlings	Micropropagation Mycorrhization <i>in vitro</i>	Strullu <i>et al.</i> (1986)
<i>C. sativa</i>	Axillary shoot cultures	Rooting Auxin protectors IAA-oxidase activity	Mato and Viéitez (1986)
<i>Castanea</i> spp.	Review	Review	Viéitez <i>et al.</i> (1986)
<i>C. sativa</i>	Shoot tips from seedlings	Micropropagation	Mullins (1987)

Continued

Table 9.1.3. *Continued.*

Species	Explants		Response /Studies	References
<i>Castanea</i> spp.	Review		Review	Schwarz (1987)
<i>C. sativa</i> ; <i>C. crenata</i> × <i>C. sativa</i>	Embryo axes Axillary buds		Micropropagation Vitrification	Piagnani and Eccher (1988)
<i>C. sativa</i> × <i>C. crenata</i>	Axillary shoots		Vitrification PGRs Endogenous cytokinins	Viéitez <i>et al.</i> (1988)
<i>C. sativa</i> ; <i>C. crenata</i>	Meristems and axillary buds from mature trees		Micropropagation	Chauvin and Saleses (1988a,b)
<i>C. sativa</i> × <i>C. crenata</i>	Shoot cultures		Micropropagation Prevention of shoot tip necrosis	Viéitez <i>et al.</i> (1989)
<i>C. sativa</i> × <i>C. crenata</i>	Axillary buds from mature trees		Rejuvenation Rooting	Ballester <i>et al.</i> (1989, 1990)
<i>C. sativa</i>	Immature embryo axes		Seedling growth Plantlets	Ivanova and Erdelsky (1989)
<i>C. sativa</i>	Axillary buds from mature trees		Rejuvenation <i>in vitro</i>	Feijó and Pais (1990)
<i>C. dentata</i>	Juvenile axillary buds		Axillary shoot proliferation Rooting	Serres <i>et al.</i> (1990)
<i>C. sativa</i> × <i>C. crenata</i>	Axillary buds from sprouts and crown branches of mature trees		Micropropagation Studies on rejuvenation	Sánchez (1991); Sánchez and Viéitez (1991)
<i>C. sativa</i> × <i>C. crenata</i>	Apical and axillary buds from sprouts		Axillary shoot proliferation Rooting and acclimatization	Miranda and Fernández (1992)
<i>C. sativa</i>	Juvenile and mature shoot cultures		SEM-TEM Leaves, glandular trichomes	Viéitez, M.L. (1992)
<i>C. sativa</i>	Shoot tips and nodes from mature trees and seedlings		Axillary shoot proliferation Rooting and acclimatization	Wilhelm and Rodkachane (1992)
<i>C. sativa</i> × <i>C. crenata</i>	Shoot tips and nodes from mature trees		Micropropagation	Gonçalves <i>et al.</i> (1993)
<i>C. dentata</i>	Axillary buds from mature trees		Axillary shoot proliferation Rooting and acclimatization	Maynard <i>et al.</i> (1993)
<i>C. sativa</i>	Nodes from mature trees		Micropropagation	Wandinger and Rodkachane (1993)

<i>C. sativa</i>	Shoot tips and nodes from mature trees	Axillary shoot proliferation Rooting	Cinelli and Pasqualetto (1993)
<i>C. sativa</i>	Juvenile and mature axillary shoots	Biochemical study Peroxidases Cytokinins	Vidal <i>et al.</i> (1994)
<i>C. sativa</i> × <i>C. crenata</i>	Shoot tips and nodes from mature trees	Rooting Acclimatization	Gonçalves <i>et al.</i> (1994)
<i>C. sativa</i> × <i>C. crenata</i>	Axillary shoot cultures	Germplasm conservation	Janeiro <i>et al.</i> (1995)
<i>C. sativa</i>	Shoot apex	Apical necrosis Micropropagation	Piagnani <i>et al.</i> (1996)
<i>C. sativa</i>	Axillary shoots	Acclimatization Ectomycorrhization	Martins <i>et al.</i> (1996)
<i>C. sativa</i>	Shoot tips and nodes from mature trees	Micropropagation Rejuvenation	Sánchez <i>et al.</i> (1997a,b)
<i>C. sativa</i> × <i>C. crenata</i>	Axillary buds from mature trees	Shoot proliferation	Szendrák <i>et al.</i> (1997)
<i>C. dentata</i> × <i>Castanea</i> sp.	Shoot tips and nodes from seedlings and mature trees	Axillary shoot proliferation and rooting Necrosis	Xing <i>et al.</i> (1997a)
<i>C. dentata</i>	Shoot tips and nodes from mature trees	Rooting Anatomy Peroxidases	Gonçalves (1998)
<i>C. sativa</i> × <i>C. crenata</i>	Juvenile and mature shoot cultures	Rooting Anatomical and biochemical aspects	Ballester <i>et al.</i> (1999)
<i>C. sativa</i> ; <i>C. sativa</i> × <i>C. crenata</i>	Juvenile and mature shoot cultures	Polyphenols	Fernández-Lorenzo <i>et al.</i> (1999)
<i>C. sativa</i>	Axillary buds from mature trees	Serial grafting/micropropagation Reinvigoration	Giovanelli and Gianini (2000)

1989; Wilhelm and Rodkachane, 1992), *C. mollissima* (Qiguang *et al.*, 1986) and *C. dentata* (Serres *et al.*, 1990; Xing *et al.*, 1997a). Propagation of unproven material is a means to increase replicates of juvenile genotypes for testing at different sites and in many environments, facilitating selection of the best genotypes and ecotypes best adapted to each region. Micropropagation also supplies a useful tool for conservation of chestnut germplasm (Janeiro *et al.*, 1995).

Micropropagation of selected genotypes of adult chestnut trees has also been described. Biondi *et al.* (1981) initiated shoot development *in vitro* from axillary buds from stump sprouts of *C. sativa*. Viéitez *et al.* (1983) reported micropropagation of adult chestnut from shoot tips and nodal segments from stump sprouts of an 18-year-old ink disease-resistant hybrid *C. sativa* × *C. crenata* selection. Rooted shoots were successfully hardened and established in soil.

Viéitez *et al.* (1986) and Schwarz (1987) reviewed the protocols for micropropagating various species and hybrids of chestnut, using juvenile and mature material, and described the media, growth regulator requirements and environmental conditions required at each stage. Explant source is crucial for successful initiation of shoot tip and nodal cultures from mature chestnut trees. The best results have been achieved from physiologically rejuvenated materials, e.g. epicormic shoots, stump sprouts and suckers (Viéitez *et al.*, 1983, 1986; Chevre and Salesses, 1985; Read *et al.*, 1985a,b; Chauvin and Salesses, 1988a,b; Sánchez, 1991). Shoot apices, axillary buds and nodal segments from new flushes in the spring, or from dormant stem segments stored at low temperatures and forced to flush either in water or in a forcing solution of 0.02% 8-hydroxyquinoline citrate, have worked well as explants (Read *et al.*, 1985b; Viéitez *et al.*, 1986). Following surface sterilization, shoot tips and nodes 5 mm long from flushed shoots (1–4 cm long) are placed vertically on establishment medium consisting of solid Gresshoff and Doy (1972) medium (GD) supplemented with 2.22 µM benzyladenine (BA). Release of phenols and tannin-like compounds from explants into the medium is a major problem for adult chestnut material at the establish-

ment stage; this can be controlled by: (i) addition of charcoal, polyvinylpyrrolidone (PVP) or ascorbic acid to the establishment medium; and (ii) transfer of explants within the first few days of culture to fresh medium or to new locations in the same culture vessel, followed by transfer to fresh medium every 2 weeks. After 6 weeks, 8–10 mm long shoot tips and nodal segments are transferred to multiplication medium consisting of GD with 0.88 µM BA, and subcultured monthly. Adventitious rooting is induced either by culture of excised shoots (2–3 cm long) for 5–7 days in medium containing 4.9–24.6 µM indolebutyric acid (IBA), or by dipping the shoot base in 2.46–4.9 µM IBA for 30–120 s. Shoots are transferred to solid GD medium without plant growth regulators (PGRs) and with one-third strength macronutrients (rooting medium). Cultures are maintained in a growth chamber with a 16 h photoperiod (30 µmol/m²/s) at 25°C day/20°C night.

Rejuvenation by partial etiolation of crown branches, grafting on to juvenile rootstocks, serial grafting and repeated cytokinin spraying of stock plants (Ballester *et al.*, 1989, 1990; Feijó and Pais, 1990; Sánchez, 1991; Sánchez and Viéitez, 1991; Sánchez *et al.*, 1997a,b; Giovannelli and Gianini, 2000) has been effective for preconditioning mature phase materials. Hyperhydricity during the multiplication stage (Viéitez *et al.*, 1985, 1986, 1988; Piagnani and Eccher, 1988) and shoot tip necrosis during rooting (Viéitez *et al.*, 1985; Piagnani *et al.*, 1996; Xing *et al.*, 1997a) can be significant problems.

The efficiency of micropropagation is genotype-dependent, but successful micropropagation from mature material has been reported for: *C. crenata* (Chevre and Salesses, 1985; Chauvin and Salesses, 1988b); *C. crenata* × *C. sativa* (Chevre and Salesses, 1985); *C. dentata* (Read *et al.*, 1985a; Maynard *et al.*, 1993; Xing *et al.*, 1996, 1997a; Szendrák *et al.*, 1997); ink disease-resistant clones of *C. sativa* × *C. crenata* (Viéitez *et al.*, 1983; Piagnani and Eccher, 1988; Sánchez, 1991; Sánchez and Viéitez, 1991; Miranda and Fernández, 1992; Gonçalves *et al.*, 1993, 1994; Sánchez *et al.*, 1997a,b; Gonçalves, 1998); and *C. sativa* (Viéitez *et al.*, 1983; Chevre and Salesses, 1985; Chauvin and Salesses, 1988b; Piagnani and Eccher, 1988; Ballester *et al.*, 1989, 1990; Feijó

and Pais, 1990; Sánchez, 1991; Cinelli and Pasqualetto, 1993; Waindinger and Rodkachane, 1993; Piagnani *et al.*, 1996; Sánchez *et al.*, 1997a,b; Giovanelli and Gianini, 2000).

Protocols developed by the Instituto Investigaciones Agrobiológicas de Galicia (IIAG) at Santiago de Compostela (Spain) and by Institut National de la Recherche Agronomique (INRA) at Bordeaux (France) have been transferred to commercial nurseries for mass propagation of selected clones of ink disease-resistant hybrids of *C. sativa* × *C. crenata* and its reciprocal *C. crenata* × *C. sativa*. In the USA, TACF is interested in micropropagating selections from its hybrid breeding programme (Read and Szendrák, 1995).

4. Somatic Cell Genetics

4.1. Regeneration

Several studies have addressed regeneration via organogenesis or somatic embryogenesis, and have utilized juvenile tissue explants. The most common *in vitro* response has been the production of rhizogenic callus (Trippi, 1963; Borrod, 1971a,b; Hebard and Kaufman, 1976, 1978; Hu and Scrivani, 1977; Keys, 1977; Viéitez *et al.*, 1978; Keys and Cech, 1979; Park and Hung, 1979; McPheeters *et al.*, 1980; González, 1981; Jeune, 1982; San-José, 1983).

4.1.1. Somatic embryogenesis

Somatic embryogenesis of *Castanea* is summarized in Table 9.1.4. Early reports described the formation of meristematic areas and embryo-like structures from cotyledons of *C. mollissima* × *C. dentata* (McPheeters *et al.*, 1980; Skirvin, 1981), *C. sativa* (González, 1981) and *C. sativa* × *C. crenata* (González *et al.*, 1985); however, such structures did not develop as somatic embryos. The production of repetitively embryogenic cultures was reported by Viéitez *et al.* (1990) for two *C. sativa* × *C. crenata* hybrids and by Merkle *et al.* (1991) for *C. dentata* in cultures initiated from immature zygotic embryos.

Induction. Induction of somatic embryogenesis has been attempted with many differ-

ent explant types and sources from various species and hybrids of chestnut. Juvenile explants have included seeds and zygotic embryos (or parts of them), explanted at various developmental stages, as well as various organs excised from micropropagated shoots, while mature explants have included developing aments, whole stamens, filaments and anthers. Viéitez *et al.* (1990) induced embryogenic cultures from immature seeds (15–20 mm long) collected approx. 8–10 weeks postanthesis from ink disease-resistant *C. sativa* × *C. crenata* clones, 'HV' and '431', on Murashige and Skoog (1962) medium (MS) supplemented with 0.45–4.52 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or with 4.44–8.88 μM BA or 4.56–9.12 μM zeatin for 2 months in darkness. They were then transferred to half-strength MS containing 0.44 μM BA with or without 0.27 μM naphthaleneacetic acid (NAA) or 0.25 μM IBA and kept under a 16 h photoperiod (30 $\mu\text{mol}/\text{m}^2/\text{s}$) with 25°C days and 20°C nights. After 2–3 months, embryogenic cultures, consisting of friable yellowish embryogenic tissue or proembryonic masses (PEMs), formed somatic embryos that were capable of regenerating plants (Viéitez *et al.*, 1991, 1992). Corredoira *et al.* (2001) induced embryogenic cultures from leaves from shoot cultures of open-pollinated seedlings of hybrid *C. sativa* × *C. crenata* on semi-solid MS with 5.37 μM NAA and 4.44 μM BA.

Embryogenic cultures of American chestnut have been induced from immature seeds on woody plant medium (WPM) (Lloyd and McCown, 1980) containing 1.11 μM BA and either 32.22 μM NAA or 18.1 μM 2,4-D (Merkle *et al.*, 1991). With European chestnut, Piagnani and Eccher (1990) induced embryogenic cultures from immature cotyledons of *C. sativa* 'G9' on half-strength MS supplemented either with 5.37 NAA alone or with 4.52 μM 2,4-D and 2.22 μM BA.

The embryogenic response appears to be related to the developmental stage of seed explants. Leva *et al.* (1993) cultured cotyledon explants of *C. sativa* 'Marrone de Greve' on semi-solid Schenk and Hildebrandt (1972) medium (SH) supplemented with 2.69 μM NAA and found that explants sampled 50 days after full blooming responded better

Table 9.1.4. Somatic embryogenesis in *Castanea*.

Species	Explants	Results/studies	References
<i>C. mollissima</i> × <i>C. dentata</i>	Cotyledon pieces	Embryoid-like protuberances	McPheeters <i>et al.</i> (1980); Skirvin (1981)
<i>C. sativa</i>	Cotyledon pieces	Embryoid-like structures	González <i>et al.</i> (1985)
<i>C. sativa</i> × <i>C. crenata</i>	Immature embryonic axes	Embryogenic callus Embryo development	Viéitez <i>et al.</i> (1990)
<i>C. sativa</i> ; <i>C. crenata</i> × <i>C. sativa</i>	Cotyledon pieces Immature embryos	Embryogenic callus Embryo development	Plagnani and Ecoher (1990)
<i>C. sativa</i> × <i>C. crenata</i>	Immature zygotic embryos	Mature somatic embryos Germination Plantlets in soil	Viéitez <i>et al.</i> (1991, 1992)
<i>C. dentata</i>	Ovules Immature zygotic embryos	Embryogenic callus Embryo development	Merkle <i>et al.</i> (1991)
<i>C. sativa</i> × <i>C. crenata</i>	Somatic embryo cultures	Cell suspension cultures Embryo production Plantlets in soil	Viéitez <i>et al.</i> (1993)
<i>C. dentata</i>	Ovules Immature zygotic embryos	Embryogenic callus Biolistic gene transfer	Merkle <i>et al.</i> (1992)
<i>C. sativa</i>	Immature zygotic embryos	Induction and embryo development	Leva <i>et al.</i> (1993)
<i>C. dentata</i>	Ovules Immature zygotic embryos	Embryogenic suspension cultures Transgenic cell lines	Carraway <i>et al.</i> (1994)
<i>C. dentata</i>	Immature zygotic embryos Embryo axes	Embryogenic cell suspensions Transgenic cell lines	Merkle and Carraway (1994)
<i>Castanea</i> spp.	Review	Review	Viéitez (1995)
<i>C. dentata</i>	Ovules	Embryo maturation	Xing <i>et al.</i> (1996)
<i>C. dentata</i>	Axes and cotyledons Immature zygotic embryos	Embryo maturation Plantlet conversion	Carraway and Merkle (1997)
<i>C. dentata</i>	Ovules Immature zygotic embryos	Transgenic cell lines Gene transfer <i>Agrobacterium</i>	Xing <i>et al.</i> (1997b)

<i>C. dentata</i>	Ovules	Transgenic cell lines	Maynard <i>et al.</i> (1998)
<i>C. sativa</i> × <i>C. crenata</i> <i>C. dentata</i>	Immature embryos	<i>Agrobacterium</i>	
	Long-term embryogenic lines	Mass balance	Viéitez (1999)
	Immature zygotic embryos	Maturation Plantlet conversion	Xing <i>et al.</i> (1999)
<i>C. sativa</i>	Ovules, ovaries	Direct embryogenesis from hypocotyls	Sauer (1999)
<i>C. dentata</i>	Immature embryos		
	Immature seeds	Cryopreservation Mature somatic embryos	Holliday and Merkle (2000)
<i>C. sativa</i>	Zygotic embryos	Mature somatic embryos Plantlet conversion	Sauer and Wilhelm (2000, 2001)
<i>C. sativa</i>	Leaf sections from juvenile clones	Induction and embryo development Incomplete germination	Corredoira <i>et al.</i> (2001)

than explants sampled later and that there were no responses 80 days after blooming. Sauer (1999) reported induction of embryogenic cultures of *C. sativa* from seeds sampled between 2 and 10 weeks postanthesis, and Sauer and Wilhelm (2000, 2001) found that induction frequency is related to the size and water content of the zygotic embryos. Explants collected 4 weeks postanthesis provided the highest induction frequencies after 8 weeks on Teasdale's (1992) P24 solid medium supplemented with 4.97 μM 2,4-D and 0.44 μM BA, but no cotyledonary embryos were produced (Sauer, 1999). Although less responsive than embryos collected earlier, the explants sampled 9–10 weeks postanthesis produced clusters of embryos that proliferated on GD or P24 medium containing 300–1000 mg/l glutamine and 0.88 μM BA (Sauer and Wilhelm, 2000, 2001).

Maintenance. Embryogenic cultures have been maintained, via secondary embryogenesis and/or by subculturing PEMs, on semi-solid medium containing cytokinins with or without auxin, generally at lower levels than for induction. Medium-term maintenance of several embryogenic *C. sativa* $\times$ *C. crenata* culture lines on semi-solid medium and as suspension cultures has been described by Viéitez (1995). Glutamine and 0.46–0.91 μM zeatin or 0.44–0.88 μM BA and 0.05–0.25 μM IBA or 0.05–0.27 μM NAA were essential for proliferation of embryogenic cultures and for sustained production of cotyledonary somatic embryos. Hybrid chestnut cultures could be maintained by monthly subculture of PEMs on semi-solid half-strength MS containing 3 mM glutamine, 0.91 μM zeatin, 0.25 μM IBA and 30 g/l sucrose under diffuse light. Sucrose at 30 g/l was superior to fructose, glucose and maltose for maintenance of PEMs and yield of cotyledonary embryos; maltose was least effective (Viéitez, 1999). After > 12 years of repeated subculture on this medium, the production of cotyledonary somatic embryos remained undiminished (Viéitez *et al.*, 1993; Viéitez, 1999). Continued proliferation of American chestnut embryogenic cultures either by secondary embryogenesis or as PEMs could

only be obtained by regular transfer to WPM containing 9.05 μM or higher 2,4-D (Merkle *et al.*, 1991; Carraway and Merkle, 1997).

Maturation. Production of hybrid chestnut cotyledonary somatic embryos varied significantly in the same manner as fresh weight (FW) gain with different carbon sources. The highest number of cotyledonary somatic embryos occurred with 30 g/l sucrose, with an average of 149 somatic embryos/g of PEMs, and approx. 33% of them show normal morphology, but with very different sizes (Viéitez, 1999). With American chestnut, total somatic embryo production was similar to the response of hybrid chestnut; however, fructose promoted higher frequencies of normal somatic embryos than 60 g/l sucrose, which in turn was superior to 30 g/l sucrose (Carraway and Merkle, 1997).

Normal morphology of developing somatic embryos is essential for recovery of mature embryos for regeneration (De Wald *et al.*, 1989; Wetzstein and Baker, 1993; Eggertsdotter, 1996; Fig. 9.1.1). Nevertheless, maturation and germination of chestnut somatic embryos has been unpredictable. Because of the lack of synchronization during somatic embryo development and the long periods required for maturation, control of development to physiological maturity has been very difficult to achieve. Furthermore, even large chestnut somatic embryos with morphologies resembling those of zygotic embryos (except for having smaller cotyledons) have regenerated plants only irregularly (Viéitez *et al.*, 1996), suggesting that they are still physiologically immature.

Maturation of chestnut somatic embryos has involved a two-step protocol: embryos are first cultured for 4 weeks on semi-solid medium with 0.44–2.22 μM BA, 0.46–2.28 μM zeatin or 0.38–37.84 μM abscisic acid (ABA) or without plant growth regulators, followed by cold stratification at 4°C of normal-shaped embryos for at least 8 weeks. Major obstacles preventing development of apparently normal somatic embryos to maturity have been callusing, development of secondary embryos, early greening, precocious germination, general overgrowth and softening or disruption of tissues, especially of those in direct



Fig. 9.1.1. Cluster of American chestnut somatic embryos at various stages of development.

contact with medium. Efficiency of maturation has been assessed as the percentage of cotyledonary somatic embryos that remain healthy and with normal morphology after each of the two stages (Viéitez, 1999). Neither 0.38–37.84 μM ABA nor 2–4% polyethylene-glycol (PEG) 8000 enhanced embryo maturation efficiency, and they caused browning and secondary embryogenesis, respectively. To date, the best results with hybrid chestnut somatic embryos have been obtained after 4 weeks on plant growth regulator-free medium with about 50% efficiency and a further 50% following 10–12 weeks at 4°C in the same plates. Subsequently, 18–20% of the somatic embryos that survived cold treatment and retained normal shapes germinated and regenerated normally on MS with 0.46–0.91 μM zeatin under a 16 h photoperiod (35–40 $\mu\text{mol}/\text{m}^2/\text{s}$). Survival after acclimatization has been approx. 80%. Some 100 hybrid chestnut somatic seedlings have been planted in soil, and all survived and displayed normal growth in the field. Many of these trees have developed male catkins as early as 2–3 years after out-planting and began to produce nuts the following season.

Sauer and Wilhelm (2000, 2001) reported that maturation of *C. sativa* somatic embryos could be enhanced significantly after 5 weeks on plant growth regulator-free P24 medium, with either 6% sorbitol or with increased (1.1%) agar concentration. After 8 weeks at 4°C, somatic embryos germinated and several plants were successfully acclimatized in the greenhouse. Corredoira *et al.* (2001) reported that approx. 6% of cotyledonary *C. sativa* $\times$ *C. crenata* somatic embryos formed shoots but no roots on MS with 0.44 μM BA following cold storage at 4°C for 8 weeks, although plants were regenerated from rooted microcuttings.

Transfer of PEMs and repetitively proliferating somatic embryos of American chestnut on to semi-solid, plant growth regulator-free WPM allowed development of somatic embryos, but germination and plant recovery were not reported (Merkle *et al.*, 1991). Culture of developing American chestnut somatic embryos on WPM supplemented with activated charcoal enhanced the yield of well-developed somatic embryos, and storage at 4°C enhanced germination, but plantlets failed to thrive following transfer to



Fig. 9.1.2. Hybrid chestnuts derived from somatic embryos after 10 years in soil.

ex vitro conditions (Carraway and Merkle, 1997). By culturing somatic embryos on B5 (Gamborg *et al.*, 1968) medium with 0.5 μM BA and 0.5 μM NAA, initially with 30 g/l sucrose for development, and then with 60 g/l sucrose for maturation, Xing *et al.* (1999) regenerated 20 American chestnut somatic seedlings, which continued to grow following transfer to potting mixture. Some of these trees have survived in the field (Maynard *et al.*, 1998; Fig. 9.1.2).

4.1.2. Organogenesis

The production of adventitious bud-like structures *in vitro* has been described several times; however, subsequent development was not observed (Table 9.1.5).

Induction. Adventitious shoot bud induction has been reported from epicotyl explants of 3–5-week-old *C. sativa* plantlets

derived from embryonic axes on semi-solid H medium (Heller, 1953) containing 4.44–8.87 μM BA (San-José, 1983; San-José and Viéitez, 1984; San-José *et al.*, 1984). For induction, 5 mm long epicotyl segments were then cultured on H medium supplemented with 8.87 μM BA alone or with 0.05 μM NAA or IBA for 4 weeks under a 16 h photoperiod (30 $\mu\text{mol}/\text{m}^2/\text{s}$) with 25°C days and 18°C nights. Adventitious buds originated in the outermost region of the cortex at the basal ends of the explants. Similarly, Seabra and Pais (1993) germinated seeds of European chestnut on a modified GD medium with 2.22–4.44 μM BA, and induced adventitious shoot buds directly from epicotyl slices cultured on GD with 4.44–6.66 μM BA. Seabra and Pais (1998) obtained similar results when hypocotyl sections of *C. sativa* seeds that germinated on MS with 2.22–4.44 μM BA were used as explants, but neither induction

Table 9.1.5. Organogenesis in Castanea.

Species	Explants	Response /Studies	References
C. sativa	Zygotic embryo axes and cotyledons	Callus and adventitious roots Histology	Viéitez et al. (1978)
C. dentata	Cotyledons and epicotyls	Adventitious bud-like structures	Keys and Cech (1979)
C. dentata; C. mollissima;	Zygotic embryo axes and cotyledons	Adventitious buds	McPheeters et al. (1980); Skirvin (1981)
C. mollissima × C. dentata			San-José (1983)
C. sativa	Epicotyls and hypocotyls from in vitro-grown seedlings	Adventitious buds and roots Plant regeneration Histology	
C. sativa	Epicotyl segments	Adventitious buds and shoots Shoot proliferation and rooting Plants in soil	San-José and Viéitez (1984); San-José et al. (1984)
C. sativa	Cotyledons, hypocotyls, roots, leaves, epicotyls	Organogenic callus Direct organogenesis from epicotyls Shoot regeneration	Seabra and Pais (1993)
C. sativa	Hypocotyls	Adventitious buds and shoots Transformation (Agrobacterium)	Seabra and Pais (1998)
C. sativa	Internodes from in vitro seedlings	Adventitious shoots	Mulin et al. (1999)
C. sativa × C. crenata	Cotyledonary nodes from juvenile clones	Adventitious buds and shoots Plant regeneration/anatomy	San-José et al. (2001)

medium nor growing conditions were detailed. Mulin *et al.* (1999) induced adventitious shoots from the epidermis of internodal longitudinal sections and internode segments from micropropagated seedlings of a *C. sativa* 'Martainha' tree after 3 weeks on MS supplemented with 6.66 μM BA and 1.14 μM indole-3-acetic acid (IAA). San-José *et al.* (2001) induced shoot organogenic cultures from cotyledonary node sections of a *C. sativa* $\times$ *C. crenata* hybrid, which contained axillary meristematic tissue. Optimum shoot production was achieved by germinating embryonic axes on MS with 4.44 μM BA (preconditioning medium) for 12–14 days and then culturing cotyledonary node explants on shoot induction medium, i.e. MS supplemented with 0.05 μM NAA and 4.54–9.08 μM thidiazuron (TDZ). After 4 weeks, 35–45% of explants produced more than ten shoot buds from each explant.

Development. Shoot bud development has been achieved by culturing the original explants on induction medium either without PGRs (San-José and Viéitez, 1984; San-José *et al.*, 1984) or with 0.04–0.20 μM BA (San-José *et al.*, 2001) to 2.22–4.44 μM BA (Seabra and Pais, 1993; Mulin *et al.*, 1999). Whole plants have been recovered by rooting shoots. Isolated shoots are either dipped in 4.90 μM IBA solution for 2–15 min (San-José *et al.*, 1984; Mulin *et al.*, 1999) or cultured on half-strength basal medium containing 9.8–14.7 μM IBA for 3–6 days (San-José *et al.*,

1984; Seabra and Pais, 1993) or 122.6 μM IBA for 24 h (San-José *et al.*, 2001) and subsequently transferred to IBA-free medium.

4.2. Genetic manipulation

The primary objective of genetic manipulation of chestnut has been the production of disease-resistant trees.

4.2.1. Mutation induction

Irradiation of American chestnut nuts has been attempted to induce mutations that would confer blight resistance. Nuts collected from nine sources throughout the range of *C. dentata* have been irradiated with different dosages of γ -rays and thermal neutrons from 1961 to 1972 (Dietz, 1978). Although thousands of M_1 and M_2 trees were produced, few results of this study have been published. Fulbright *et al.* (1992) reported that some of the surviving trees derived from irradiated nuts showed increased resistance in the form of non-lethal cankers following inoculation with a virulent strain of the blight fungus.

4.2.2. Genetic transformation (Table 9.1.6)

Efficient gene transfer for chestnut would allow genes with potential antifungal activity to be tested; however, the lack of reliable *de novo* regeneration for *Castanea* has severely limited such studies. Carraway *et al.* (1994)

Table 9.1.6. Genetic transformation in *Castanea*.

Species	Approach	References
<i>C. dentata</i>	<i>Agrobacterium</i> ; PCR	Maynard (1991b)
<i>C. dentata</i>	Biolic gene transfer of embryogenic cell suspension	Merkle <i>et al.</i> (1992)
<i>C. sativa</i>	<i>Agrobacterium rhizogenes</i> -mediated rooting of epicormic shoots	Rinallo and Mariotti (1993a,b)
<i>C. dentata</i>	Biolic transformation of embryogenic cultures	Merkle and Carraway (1994)
<i>C. dentata</i>	Biolic transformation of embryogenic cultures	Carraway <i>et al.</i> (1994)
<i>C. dentata</i>	<i>Agrobacterium</i> -mediated transformation of embryogenic cultures	Xing <i>et al.</i> (1997b)
<i>C. dentata</i>	<i>Agrobacterium</i> -mediated transformation of embryogenic cell lines	Maynard <i>et al.</i> (1998)
<i>C. sativa</i>	<i>Agrobacterium</i> -mediated transformation of hypocotyls; transgenic adventitious shoots	Seabra and Pais (1998)

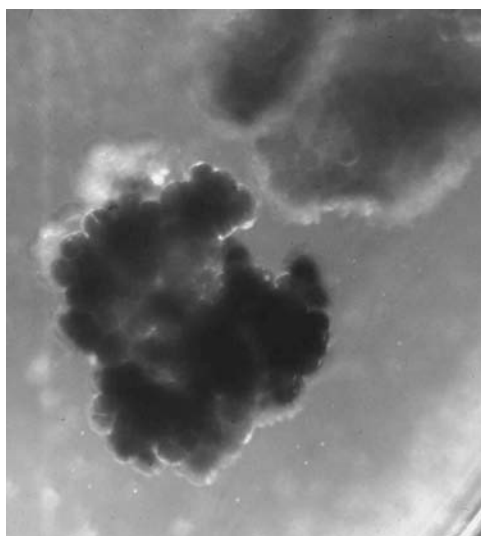


Fig. 9.1.3. American chestnut cell clump transformed via microprojectile bombardment expressing the β -glucuronidase (GUS) gene.

produced transgenic American chestnut lines following microprojectile bombardment of embryogenic cultures using the plasmid pBI121, which carried genes for the selectable marker neomycin phosphotransferase (*NPTII*) and the β -glucuronidase (GUS) reporter (Fig. 9.1.3). Although the transformed cells were shown to have integrated the transferred DNA and expressed the GUS gene, no somatic embryos were produced. Embryogenic American chestnut cultures have been transformed using *Agrobacterium* with unnamed marker genes and the antimicrobial peptide gene ESF12, under the control of a poplar wound-inducible promoter, although transgenic plants were not regenerated (Maynard, *et al.*, 1998). *Agrobacterium*-mediated transformation of hypocotyl tissue has also been used to produce shoots transformed with the plasmid p35SGUSINT carrying the *NPTII* and GUS genes, although whole plants were not regenerated (Seabra and Pais, 1998).

4.3. Cryopreservation

Conservation of chestnut genetic diversity is crucial for cultivar development and to protect disease-threatened wild popula-

tions. The multiple population breeding strategy (Eriksson *et al.*, 1993) has been recommended in Europe for conservation of genetic diversity in wild populations, while the cultivated chestnuts should be conserved in clonal archives (Eriksson and Fernández, 2001). Conservation of chestnut in field gene banks is threatened by pests, diseases and abiotic risks. Cryopreservation is the only method currently available to ensure the safe and long-term conservation of genetic resources of species like chestnuts that have recalcitrant seeds and/or are vegetatively propagated (Engelmann, 2000).

Pence (1990, 1992) showed the feasibility of cryopreserving isolated embryo axes of *C. mollissima* and *C. sativa*. Although surviving embryos did not develop sufficiently to regenerate plants, the highest survival rates were achieved with axes that were desiccated to about 20–30% moisture content (Pence, 1992). Holliday and Merkle (2000) successfully cryostored and recovered embryogenic *C. dentata* cultures using sorbitol for osmotic pre-treatment and dimethyl sulphoxide (DMSO) for cryoprotection. Following cryostorage, embryogenic cultures proliferated and somatic embryos were recovered. At the IIAG, cryogenic storage of various chestnut tissues has been facilitated by preconditioning cultures on high sucrose media and applying standard vitrification treatments. Embryo axes from *C. sativa* and *C. sativa* $\times$ *C. crenata* mature seeds, shoot tips and axillary buds from vigorously growing axillary shoot cultures (Fig. 9.1.4) and embryogenic cultures of hybrid chestnut have all survived cryogenic storage (M.C. San-José, IIAG, Santiago de Compostela, Spain, personal communication).

5. Conclusions

Chestnuts are one of the most important nut crops; however, loss of trees, particularly due to fungal diseases, has created a worldwide shortage of chestnuts. Biotechnology offers a set of very valuable tools that can help meet the increasing demand for chestnuts and guarantee the sustainability of chestnut-based industries. Various *in vitro* protocols,

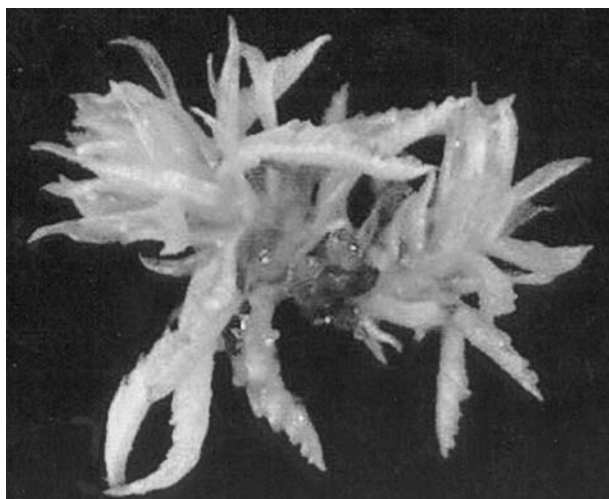


Fig. 9.1.4. Shoot culture derived from cryostored shoot tips of ink disease-resistant *Castanea sativa* × *C. crenata*.

genome analysis, marker-assisted selection and genetic engineering provide opportunities to overcome the genetic features of chestnuts that limit conventional breeding methods. Chestnut breeding programmes are well established, and production of new, superior chestnut cultivars must also rely on conventional breeding. Genotype × environment interaction dictates that field testing should be accomplished at different locations and over several years to determine the best selections and which genotypes will be included as parents in advanced breeding programmes. Molecular markers are useful for establishing variation within and among *Castanea* species, and may accelerate breeding through marker-assisted selection.

Standard micropropagation is promising, but progress with *de novo* regeneration has lagged, delaying the application of somatic cell genetics to the genus. As demand for the crop continues to outpace supply, more resources will be devoted to developing disease-resistant, high-yielding *Castanea* clones.

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10

Juglandaceae

The family *Juglandaceae* includes about 50 species in eight genera of deciduous, monoecious trees or shrubs with alternate, pinnately compound leaves (Watson and Dallwitz, 1992 onwards). Constituent species are divided into the genera *Juglans* L. (walnuts), *Carya* Nutt. (pecans and hickories), *Pterocarya* Kunth. (wingnuts), *Platycarya* Sieb.

and Zucc., *Engelhardia* Lesch. Ex Blume, *Alfaroa* Standl. and *Oreomunnea* Oerst. A comprehensive phylogenetic analysis using morphological, chemical, chromosomal and DNA sequence-based markers has been carried out to investigate the evolution, phylogeny and sytematics of *Juglandaceae* (Manos and Stone, 2001).

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10.1 *Carya illinoensis* Pecan

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1. Introduction

1.1. Botany and history

Pecan is a diploid ($2n = 2x = 32$) deciduous tree within the genus *Carya* (*C. illinoensis* (Wangenh.) K. Koch). There are approx. 20–25 species of large trees in this genus. The leaves are alternate and compound, varying in size, e.g. ‘Cheyenne’ has small leaves, while ‘Mahan’ has large leaves. Leaflets are usually 5–10 cm in length and vary from nine up to 17 leaflets per leaf. Leaf colour is variable, from the yellow-green of ‘Desirable’ to the extremely dark leaves of ‘Pawnee’ (Reed and Davidson, 1954; Sparks, 1992).

Pecan is a monoecious tree with staminate (male) and pistillate (female) flowers on the same tree. Pistillate flowers are borne in terminal spikes on new shoot growth, while pendulous staminate inflorescences are borne on the base of the shoot and along the length of the supporting 1-year-old wood (Wetzstein and Sparks, 1983; Sparks, 1992). Staminate flowers differentiate during spring on current season growth, with pollen shedding the next spring, whereas pistillate flowers differentiate at the time growth resumes in spring (Wetzstein and Sparks, 1983, 1984). Large amounts of pollen are produced, enhancing pollination by wind. Dichogamy is present, defined as different times of anther dehiscence and pistil receptivity.

Pecan is heterodichogamous, with protandrous (pollen dehiscence prior to stigma receptivity) or protogynous (stigma receptivity prior to pollen dehiscence) flower habits in different genotypes. Dichogamy usually promotes cross-pollination, although a brief period of overlap in some cultivars may favour self-pollination. Since geographical location and year-to-year variations can affect dichogamy, a proper selection of cultivars must be chosen for proper pollination (Sparks, 1992).

The pecan nut (or fruit) is actually the kernel enclosed by the shell. The nut is enclosed by the shuck that originates from the floral involucre, which dries and opens at maturity, splitting away from the nut in four valves. Fruit maturation is achieved in the autumn of the same year and it is directly related to the cultivar and its region of origin (Peterson, 1990; Sparks, 1992).

Pecan is native to the Mississippi River valley, with its native habitat extending from south-west Texas to south-east Alabama, north to southern Illinois and south to the mountains of northern Mexico.

1.2. Importance

Pecan is the most widely cultivated and the most economically important member of the

North American hickory group. Beyond its native range, pecan is commercially grown from the south-eastern USA to the south-west (California) (Hanna, 1987; Peterson, 1990; Harlow *et al.*, 1991). Commercial production also occurs in Australia, Brazil, Canada, Israel, Mexico and South Africa, although the USA is the leading producer. Georgia is the leading state for pecan production, representing about 30% of the total USA production.

The nut is the main product of economic importance, although other uses include wood products for flooring, furniture and veneer, a natural habitat and food source for wildlife, and as an ornamental tree. In addition, the oil extracted from the kernel is edible and is used for the production of drugs and essential oils.

1.3. Breeding and genetics

The first written description of pecan dates from 1520 and is by the Spanish explorer Cabeza de Vaca. Thompson and Young (1985) indicate that pecan was first brought into cultivation in 1766. Pecan cultivars date from 1846–1847, when ‘Centennial’ was selected and propagated at the Oak Alley Plantation in Louisiana, USA. Pecan is usually propagated by budding or grafting improved cultivars on to open-pollinated seedling rootstocks. Rootstock improvement strategies have not been developed for pecan and currently there is no commercial production of clonal rootstocks.

Pecan cultivars originate from three sources: chance seedlings, selections from either seedling orchards or ‘dooryard’ seedlings and breeding programmes. Cultivar selection either from ‘dooryard’ seedlings or from seed orchards has been the major source of commercially important cultivars. Cultivar selection by breeding was initiated in the early 20th century, mainly by programmes conducted by state or federal research institutions (Sparks, 1992).

1.3.1. Breeding objectives

Major breeding objectives in pecan include several horticulturally important traits, e.g.

tree characteristics, fruit and nut characteristics and resistance to insects, diseases and winter injury. Tree characteristics include time of bud break, fruiting habit, dichogamy, leaf size, orientation, colour and retention, tree structure, size and shape; alternate bearing, precocity and prolific bearing, cluster size; length and density of fruiting shoots; and fruit retention. Important fruit and nut characteristics are time of nut maturity, shuck characteristics, shell markings, nut size and shape, kernel colour, grooves, plumpness and oil percentage, shell thickness; and adaptability to mechanical harvesting, ease of mechanical cracking and shelling and storage ability. Insect pests include aphids, bud moth, leaf phylloxera, case-bearer, bark beetle and borers; however, insect resistance is not a primary consideration for cultivar selection. On the other hand, disease resistance, primarily resistance to leaf and fruit scab, is a major factor for cultivar selection. Resistance to winter injury is also important because it determines the geographic range for growing a particular cultivar (Sparks, 1992). The identification of seedling pecan lines with superior rootstock characteristics, i.e. lack of variability, vigour and improved yield of the scion, has been unsuccessful (Hanna, 1987).

1.3.2. Breeding accomplishments

Pecan breeding programmes have emphasized the development of precocious and prolific cultivars. This is due to the inherent low yield of pecan, and non-precocious cultivars prolong the slow return; however, prolific cultivars resulting from such breeding programmes have resulted in selections whose nuts fail to fill in mature trees. On the other hand, prolific cultivars with good nut quality on mature trees produce unacceptably small nuts. The ideotype cultivar is ‘Desirable’, which produces a small number of fruits per cluster and has good to excellent nut quality and return bloom (Sparks, 1992).

Forkert (1914) and Risien (1914) initiated pecan breeding, although their breeding techniques and therefore the parentages of their cultivars are still questionable (Sparks, 1992). According to Crane *et al.* (1938), the

North Carolina Agricultural Experiment Station established a breeding programme in 1912; however, only a single cultivar, 'Cape Fear', was released. A breeding programme was established by the US Department of Agriculture (USDA) at approximately the same time, but was discontinued during the First World War. The programme was resumed in 1920 in Georgia, but was later discontinued, after only one cultivar, 'Woodroof', was released (Daniell *et al.*, 1983). Between 1931 and 1964, L.D. Romberg of the Texas Department of Agriculture released 18 pecan cultivars.

Following the development of cultivars for the southern states of the USA, several superior cultivars were developed for intermediate season states by the Oklahoma Agricultural Experiment Station, with emphasis on early ripening, resistance to scab, ease of shelling and kernel percentage. Seven cultivars were released from this programme. New Mexico released four cultivars adapted to high elevations. The development of northern cultivars started in 1908, and the focus has been on developing cultivars with fruit maturation in a short growing season. Over 30 cultivars have been released from this programme (Sparks, 1992).

2. Molecular Genetics

There have been no reported studies involving gene cloning and the use of molecular tools for marker-assisted selection. The use of molecular markers for characterizing somaclonal variants is discussed below.

3. Micropropagation

Wood (1982) and Knox and Smith (1981) attempted to establish proliferating shoot cultures from nodal stem segment explants from seedlings. Although normal shoot elongation was obtained, the root system was poor and no plants were established in soil. Phillips and Ramirez (1983), Ramirez-Martinez (1983) and Cortes-Olivares *et al.* (1990) obtained successful shoot elongation using apical and axillary buds from mature

trees, but rooting and establishment of plantlets were unsatisfactory. Cortes-Olivares *et al.* (1990) obtained 40% rooting of shoots. Hansen and Lazarte (1984) used nodal sections from seedlings as explants, and observed limited shoot multiplication; shoots were rooted with 93% success and plants were established in soil. Yates and Wood (1989) obtained plants using embryonic axes from developing seeds as explants. Obeidy and Smith (1990, 1993) addressed the problem associated with fungal contamination by manipulating the water availability of the medium and established plantlets in soil. Micropropagation has limited utility due to poor rooting, high rates of contamination and low regeneration.

4. Somatic Cell Genetics

4.1. Regeneration

4.1.1. Somatic embryogenesis

Somatic embryogenesis is a potential method for clonal propagation of pecan rootstocks for characteristics such as dwarfing for size control, enhanced nutrient uptake, alternate bearing control, salinity tolerance, nematode resistance and growth uniformity. Furthermore, since embryogenic cultures are amenable to genetic transformation, other important cultivar characteristics could be enhanced, such as disease and insect resistance. These strategies could be incorporated into a pecan improvement programme (Wetzstein *et al.*, 2000).

Somatic embryogenesis has been developed and improved for pecan (Merkle *et al.*, 1987; Wetzstein *et al.*, 1989, 1990; Mathews and Wetzstein, 1993). Unlike plants regenerated via the organogenic pathway, somatic embryo-derived plants exhibit root morphology that is comparable to that of seedlings. The repetitive nature of embryogenic cultures makes this model very attractive for mass clonal propagation and for genetic transformation studies (Wetzstein *et al.*, 1996).

Induction. Immature zygotic embryos are utilized as the primary explants for induction of embryogenic cultures. The develop-

mental stage of the zygotic embryo is critical, and approx. 15 weeks after pollination is the optimum stage. This stage of development is characterized by rapid cotyledon expansion and the presence of liquid and gelatinous endosperm. The shell hardening is at two-thirds of the total length at this stage (Wetzstein *et al.*, 1989). A semi-solid induction medium, woody plant medium (WPM) (Lloyd and McCown, 1980), supplemented with 30 g/l sucrose, 1 g/l casein hydrolysate, 1.2 μ M benzyladenine (BA) and either 32 or 64 μ M naphthaleneacetic acid (NAA) is used. After culture on induction medium for 1 week, the explanted embryos are transferred to WPM without growth regulators in the dark at 30°C, where somatic embryo development occurs. One week on induction medium is sufficient for a good embryogenic response; however, the response is genotype-dependent and varies among cultivars (Yates and Reilly, 1990).

Maintenance. Embryogenic cultures are maintained by the repetitive proliferation of secondary embryos from globular and early stage somatic embryos following their transfer to basal medium without growth regulators. Embryogenic cultures are subcultured at 3-week intervals on to modified basal WPM, and maintained in the dark at 27 $\pm$ 2°C. Some embryogenic cultures have been maintained for > 8 years (H.Y. Wetzstein,

personal communication) without loss of embryogenic competence. Rodriguez and Wetzstein (1994) described globular, heart shape and cotyledonary stages of somatic embryos in repetitive embryogenic cultures. Somatic embryos with clear bipolarity and well-developed shoot apices and cotyledons can be harvested at any time for plant regeneration.

Development/maturation. After shoot and root apices have differentiated from well-developed somatic embryos with cotyledons, individual somatic embryos are isolated for enlargement on fresh WPM basal medium (Fig. 10.1.1). After the embryos reach 8–10 mm width, they are submitted to conversion enhancement treatments to facilitate plant recovery. Such treatments are necessary to prevent them from dedifferentiating and forming new somatic embryos or callus (Mathews and Wetzstein, 1993).

Germination/conversion. Wetzstein *et al.* (1989) tested various desiccation treatments to promote conversion of somatic embryos and found that longer desiccation treatments (5 days) improved root growth. Embryo conversion was further improved by a cold treatment (5°C for 5 weeks) followed by 5 days' desiccation. Mature somatic embryos must undergo a conversion treatment to enhance germination; otherwise dedifferenti-

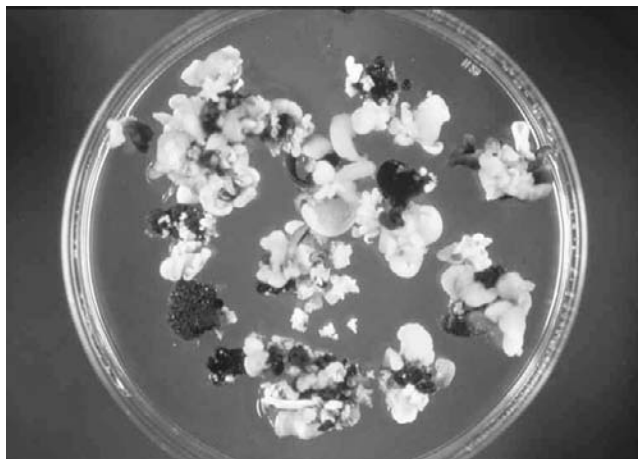


Fig. 10.1.1. Somatic embryos of pecan during maturation.

ation may occur (Mathews and Wetzstein, 1993). Somatic embryos are held at 5°C for 5 weeks and then desiccated for 1 to 5 days to promote conversion into plantlets (Wetzstein *et al.*, 1990). The combination of both treatments improved root emergence significantly (72%), and this was essential for establishment in potting mixture, since shoot development after germination limited high plant survival. Maturation of zygotic embryos is characterized by an exponential increase in embryo weight and a concomitant decline in water content. Storage deposition takes place (Jeyaretnam *et al.*, 1999). A large portion of pecan zygotic embryos (68%) is comprised of lipids (Rudolph *et al.*, 1992), of which > 98% are storage lipids, with triglycerides as the major components. Triglycerides are converted into fatty acids during germination. Somatic embryos have lower triglyceride content per embryo and per unit weight compared to zygotic embryos. Conversion enhancement treatments increase significantly the triglyceride content in somatic embryos and therefore higher levels of unsaturated fatty acids are observed. Likewise, the treatments also increase the total protein content to a level similar to that in zygotic embryos. Further conversion improvement was obtained by Mathews and Wetzstein (1993) by adding 5

mg/l silver nitrate to the germination medium (WPM) with additional application of BA (100 μ M) directly to the shoot apex of somatic embryos. Silver nitrate may inhibit ethylene biosynthesis. The application of BA directly to the shoot apex eliminates the detrimental effects that this cytokinin may have on rooting. These treatments followed the cold and desiccation treatments. Conversion rates can be increased to 20%, and approx. 70–80% of the plantlets have been hardened off successfully. Almost all of the hardened plants have been successfully acclimatized under greenhouse conditions (Fig. 10.1.2).

4.2. Somaclonal variation

Phenotypic analysis is the simplest and easiest method for evaluating somaclonal variation. Other common techniques include chromosome analysis, protein electrophoresis and DNA restriction fragments (De Klerk, 1990). Pecan trees regenerated via somatic embryogenesis have been field-tested for clonal fidelity (Vendrame *et al.*, 2000). Besides phenotypic evaluation, molecular analysis has also been performed. Two different culture lines were represented in the field, and both phenotypic and molecu-



Fig. 10.1.2. Acclimatized pecan plantlets derived from somatic embryos.

lar analyses were performed with somatic embryo-derived trees for differences within and between culture lines. Trees were approx. 3–4 years old at the time of evaluation. Phenotypic analysis consisted of general growth measurements, leaf morphological characteristics and pest susceptibility. All trees derived from embryogenic cultures showed vigorous growth. No significant differences were observed in comparisons among trees from the two lines for tree height, total shoot growth, trunk caliper and shoot length per trunk cross-sectional area. Differences in branching habit were observed, which were consistent with differences between the two cultivars from which the lines were initiated. Branching, branch angle, shoot growth and the degree of apical dominance were demonstrated to be under genetic control by Kramer and Kozlowski (1979). The extensive number of shoots observed in one somaclone corresponds to characteristics for 'Mahan', the maternal parent, described by Sparks (1992) as having a multiple branching habit.

Differences were significant between the two lines for susceptibility to scab (*Cladosporium caryigenum*). However, long-term evaluations are needed to determine the extent of scab resistance. The presence of phylloxera galls (*Russellae stuetzel*) was also evaluated and no significant differences were observed between the two lines.

The genetic variability in pecan embryogenic cultures and in field-grown somatic embryo-derived pecan trees (Vendrame *et al.*, 1999) was analysed using amplified fragment length polymorphism (AFLP). Differences observed within culture lines were confirmed with the replication of the experiment. A total of 361 polymorphic fragments were identified using three primer pairs.

Trees regenerated from somatic embryos appeared to be phenotypically stable. After 4 years in the field, no marked differences or aberrant variations were observed. Polymorphisms shown by AFLP analysis were apparently not reflected in the phenotype and may be related to later stages of development, i.e. flowering, fruiting, etc.

4.3. Genetic transformation

Genetic transformation offers great potential for pecan breeding programmes. Genes for scab resistance, early nut maturity, etc. could be isolated and inserted into problematic cultivars. However, there is little information on the pecan genome (Thompson and Romberg, 1985; Marquard, 1991). Genes for specific traits of economic importance need to be identified before a genetic transformation programme can be developed for pecan. The combination of genetic transformation with *in vitro* protocols is an attractive strategy for obtaining clonal rootstocks of pecan with superior characteristics.

Somatic embryogenesis is amenable to genetic transformation. The repetitive nature of somatic embryogenesis in pecan allows continued high-frequency embryogenesis and long-term regeneration of transformed lines (Wetzstein *et al.*, 1996). Burns *et al.* (1991) studied levels of kanamycin for a selection of cultures bombarded with foreign DNA. He used a PBI121.1 construct containing the cauliflower mosaic virus (CaMV) 35S promoter upstream of the β -glucuronidase (GUS) gene, a 5' nos-driven neomycin-phosphotransferase II (NPTII) gene and a gene for kanamycin resistance for selection. Although stable GUS expression was obtained, variation in gene expression and the presence of chimeric embryos contributed to the lack of success. *Agrobacterium*-mediated transformation has also been attempted (McGranahan *et al.*, 1993). *Agrobacterium* strain EHA 101/pCGN 7001 containing marker genes for kanamycin resistance (APH(3')II) and GUS was used. The construct also contained a CaMV 35S promoter, tn73', the 3' polyadenylation site of transcript 7 of *Agrobacterium tumefaciens*, and the mannopine synthase (mas) gene. Somatic embryos were co-cultivated with *Agrobacterium*, using GUS expression and kanamycin resistance as indicators of successful transformation. Although transgenic clones were obtained, plant regeneration was limited.

Suspension cultures are well suited for genetic transformation and mass production of synchronized somatic embryos. The use of suspension cultures in selection protocols

prevents the formation of chimeras (Mathews *et al.*, 1992). This might be the next step for the development of a successful transformation system in pecan.

4.4. Cryopreservation

Limited cryopreservation studies in pecan have been attempted for germplasm preservation. Morrissey (1990) studied the effect of dehydration for cryopreservation of buds of pecan and Abou Taleb *et al.* (1992) evaluated cryogenic storage of zygotic embryos and subsequent *in vitro* plant regeneration. Both studies have not been followed through and no additional information is available.

5. Conclusions

Pecan somatic embryogenesis from zygotic embryos is a very efficient regeneration system. Field tests have demonstrated good performance of somatic embryo-derived trees in terms of tree form, growth and genetic stability. Additional studies, including reproduc-

tive characteristics, e.g. flowering, fruit formation, development and bearing, will confirm the genetic fidelity of trees regenerated from somatic embryos. Challenges for the future include the establishment of embryogenic cultures from mature phase tissues, which would allow the clonal propagation of improved genotypes. Further identification of promising embryogenic genotypes and the use of genetic transformation techniques are also potential applications for crop improvement. The identification of genes of economic importance for pecan, i.e. disease and insect resistance, increased yield, nut quality, etc., will be essential for the development of new breeding programmes. The combination of *in vitro* systems, molecular biology and genetic transformation protocols will contribute to an ultimate strategy for pecan breeding and crop improvement.

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10.2 *Juglans regia* Walnut

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1. Introduction

1.1. Botany and history

The English or Persian walnut (*Juglans regia* L.) is the most horticulturally developed and widely cultivated of all the walnut species (*Juglans* L.). The genus *Juglans* consists of four sections. Three of these, *Rhysocaryon* (black walnuts native to the Americas), *Cardiocaryon* (Japanese, Manchurian and Chinese walnuts, including selections known as heartnuts) and *Trachycaryon* (the butternut of eastern North America), exhibit thick shells and non-dehiscent hulls (Manning, 1978). The fourth section, *Juglans*, is comprised of a single species, *Juglans regia* L., distinguished by a dehiscent hull which separates from the shell at maturity (Manning, 1978). Trees are monoecious with male flowers borne in catkins and female flowers in pistillate spikes of mostly two but sometimes as many as five flowers at the tips of terminal or lateral shoots. Flowering is dichogamous with either the male (protandry) or female (protogyny) flowers maturing first. Pollen is wind-borne and the dichogamy promotes outcrossing (Forde and Griggs, 1972).

J. regia, the Persian walnut, is native to central Asia and grows as a wild or semi-cultivated tree in a wide area from south-eastern Europe and the Caucasus to Turkey and

Iran, through southern portions of the former Soviet Union into China and the eastern Himalayas. It has been cultivated for its nut crop for at least several thousand years and was probably introduced into European commerce and agriculture by the ancient Greeks. It was prized by the Romans as *Jovis glans* and was utilized in medieval Europe as a herbal medicine, particularly for brain and scalp ailments. Since its introduction into North America it has commonly been referred to as the English walnut to distinguish it from the American black walnut (Leslie and McGranahan, 1998).

Grafting techniques developed in France allowed the first selection, development and propagation of cultivars. Many of these were introduced into California, USA, in the late 1800s where seedling orchards derived from introduced Spanish and Chinese seed sources had been previously established. In ensuing years, selections of superior seedling trees found in orchards derived from these combined sources of materials were propagated to form the basis of the California walnut industry (McGranahan and Leslie, 1990; Forde and McGranahan, 1996; Ramos, 1998).

Successful implementation of grafting allowed not only the development of improved cultivars, but also a choice of rootstock. In much of the world, *J. regia* seedlings are used as rootstock but in California the

native black walnut species, *J. hindsii*, has been widely preferred for its enhanced vigour, salt tolerance and disease resistance. In the early 1900s, Luther Burbank first observed the superior vigour of *J. hindsii* × *J. regia* hybrids, which he named 'Paradox' (Fig. 10.2.1; Whitson *et al.*, 1914; Howard, 1945). Most California walnut orchards are currently grown on either seedling 'Paradox' or seedling *J. hindsii* rootstock. Development of clonal rootstock has been impeded by the difficulty of rooting walnut cuttings.

1.2. Importance

Walnuts are now a global crop. Major walnut-producing regions of the world are the USA, with about 294,830 t, Europe, with 202,000 t, and China, with 360,000 t. Areas where walnuts were once native are increasing their production and new industries are rapidly developing in South America and South Africa, as well as Australia and New Zealand. Yield per ha can be as high as 3.6 t with new cultivars, although a good yield is 1.8–2.7 t. Production in the USA occurring on over 75,000 ha accounts for one-fifth of the world's \$1.2 billion production (FAO, 2004).

1.3. Breeding and genetics

Like many seeded plants, the history of walnut breeding began through chance

selection of superior nuts and seedlings. Walnuts ($2n = 2x = 32$) were typically grown from seed in most parts of the world and it is only fairly recently that they have been propagated by grafting. In California, USA, walnut cultivation dates back to the Spanish settlers, who were probably the first to grow Persian walnuts. These 'mission walnuts' were typically small with hard shells. In 1867 Joseph Sexton obtained a bag of nuts from Chile and planted them in the coastal areas of southern California. From these trees various selections were made that ultimately resulted in a seedling type known as 'Santa Barbara soft shell' walnuts. Many were named and propagated as cultivars, e.g. 'El Monte', 'Wasson', 'Ehrhardt' and 'Placentia'. In 1871 nurseryman Felix Gillet imported many of the French varieties. Both seedlings of these and grafts of the originals were planted extensively in northern California. Several older varieties, such as 'Payne' and 'Hartley', were selected from these seedlings in northern California.

A formal breeding programme was initiated at the University of California at Davis in the late 1940s under the direction of Gene Serr and Harold Forde. After Gene Serr retired in 1965, the breeding programme was continued under Harold Forde until 1978. This was a very successful partnership, which resulted in the release of 15 cultivars (Serr and Forde, 1968; McGranahan *et al.*, 1990b, 1992; Tulecke and McGranahan, 1994). Currently, the breeding programme continues under the direction of Gale McGranahan.

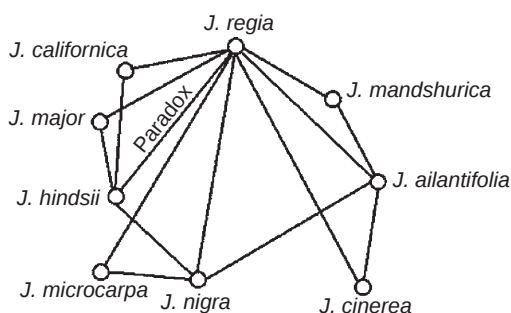


Fig. 10.2.1. Hybrids between *Juglans* species indicated by the connecting lines. Paradox, a prominent rootstock, is a hybrid between *J. regia* and *J. hindsii*. (Adapted from Funk, 1979; McGranahan and Catlin, 1987.)

1.3.1. Rootstocks

Nearly all walnut production in the USA, and increasingly worldwide, is derived from grafted scion varieties on seedling rootstocks. This reflects the difficulty of rooting walnuts, precluding, until recently, substantial use of either rooted scions or improved clonal rootstocks. Walnut production in many parts of the world today and in California during the late 1800s utilized seedlings of *J. regia* as rootstocks. Employment of rootstocks is driven by the need to deal with soil, environment, disease and pest problems (McGranahan and Catlin, 1987). In California *J. hindsii* (northern California black walnut) was preferred for much of the first half of the 20th century as it was a native species and adapted to a number of soil-related problems including resistance to crown and root rot caused by *Armillaria mellea* (oak root fungus) and tolerance of waterlogging and drought (Smith *et al.*, 1912). Today the most popular rootstocks are the interspecific hybrids, the first of which was 'Paradox', a hybrid between *J. hindsii* and *J. regia* described in 1893 by Burbank (Fig. 10.2.1; Whitson *et al.*, 1914; Howard, 1946).

Major breeding objectives. The major objective for walnut rootstock breeding is vigour, in order to promote rapid growth of the scion under a variety of soil and environmental conditions and to quickly establish a full-sized bearing canopy. Other objectives include resistance to disease and pests, most notably *Phytophthora*, nematodes and crown gall, tolerance of cherry leafroll virus, and tolerance of soil-related problems including waterlogging, salt accumulation and cold. There is interest in controlling tree size but not at the cost of vigour.

Breeding accomplishments

Vigour. A key component of orchard profitability is early return on a grower's investment in a new orchard establishment. Growers seek to achieve rapid canopy development. Early work by Luther Burbank and others identified the hybrid vigour of *J. hindsii* × *J. regia* seedlings. Figure 10.2.1 shows the possible natural hybrids that occur

among *Juglans* species. 'Paradox' rootstocks, which are very large vigorous trees themselves, showed their potential in early field studies (Serr and Forde, 1951). A major limiting factor to early widespread commercial use was limited availability due to the low incidence of 'Paradox' seeds obtained from most open-pollinated *J. hindsii*. Nurseries have now identified parent trees that produce a higher percentage of 'Paradox' seeds, but it has also become clear, using genetic analysis, that the name 'Paradox' has been applied quite loosely to describe any progeny obtained by crossing *J. regia* with any black species. This was highlighted recently in the Paradox Diversity Study conducted to characterize all of the 'Paradox' seedling sources in use by the walnut industry in California (Potter *et al.*, 2002b).

Phytophthora. *Phytophthora* is the most significant root disease of walnut in California (Mircetich and Matheron, 1983). Initial symptoms include poor growth, premature senescence in the scion, twig dieback, small chlorotic leaves, partial defoliation and finally death. In the rootstock one can observe death of the small secondary feeder root system and cankers in the crown area, which elongate up towards the stem. There are at least eight species that have been implicated in the decline of walnut trees, including *P. citricola*, *P. cactorum*, *P. cryptogea* and *P. cinnamomi*. These four species can be grown or isolated from other plant species and retain their pathogenicity on walnuts, indicating innumerable sources of inoculum for this disease in California. The disease is easily spread by irrigation water and periodic winter flooding. Where this has occurred it is not unusual for entire orchards to be affected by the disease. In these circumstances one is able to identify occasional healthy trees, especially because seedling rootstocks are used. The major limitation of capturing this potential resistance to *Phytophthora* is the difficulty in propagating such chance trees to conduct further testing and for subsequent commercialization.

Wingnut is very resistant to these strains of *Phytophthora*, but when used as a rootstock it is graft-incompatible with some cultivars

of *J. regia*. Hybrids between walnut and wingnut could potentially be used as long as they are compatible and retain the resistant trait, but hybrids developed though controlled pollination and embryo rescue have not survived over winter in the field (McGranahan *et al.*, 1986).

Tolerance of cherry leafroll virus. Cherry leafroll virus (CLRV) is the major viral disease of walnuts. The disease is spread by pollen (Massalski and Cooper, 1984) and is systemic and virtually asymptomatic in *J. regia*. Most black and 'Paradox' rootstocks are resistant to this virus and prevent the systemic spread by inducing a hypersensitive response, which is manifested as a black line at the graft union (Mircetich and Rowhani, 1984). This hypersensitive response results in the death of a cell layer at the graft union, which in turn causes a lethal girdle resulting in the decline, dieback and death of the scion (Mircetich *et al.*, 1980). The disease is asymptomatic as it travels through the scion and can only be detected by an enzyme-linked immunosorbent assay (ELISA) (Rowhani *et al.*, 1985) or reverse transcription-polymerase chain reaction (RT-PCR) (Borja and Ponz, 1992). Blackline disease was first observed in Oregon, USA, in 1925 and has spread to most growing regions in California. This disease is also prevalent in France, Italy, Hungary and the UK. Using a tolerant rootstock like *J. regia* is an option that has been tried in Oregon, where growers employ the cultivar 'Manregian'. The problem is that most English walnuts perform poorly as rootstocks due to their susceptibility to other diseases and to soil-related problems (McGranahan and Catlin, 1987).

'Paradox' $\times$ *J. regia* rootstocks that combine both the vigour of 'Paradox' and tolerance to CLRV have been selected for testing as an alternative to *J. regia*. Another course would be to develop English rootstocks that are resistant to soil pest and pathogen problems.

Resistance to nematodes. Nematodes are a major problem in California and include root lesion nematode (*Pratylenchus vulnus* Allen and Jenson), ring nematode (*Cricanemella xenoplax* Raski), root knot nematode

(*Meloidogyne* spp.) and dagger nematode (*Xiphinema americanum* Cobb). Typical scion symptoms are poor growth and dieback resulting from lesions and galls on the young feeder roots. The most significant nematode pest, *P. vulnus*, is very widespread in California and is highly pathogenic. Soil conditions influence the type of nematodes encountered. For example, sandy soils cause a build-up of root knot and ring nematodes. California black walnut (*J. hindsii*) is resistant to the root knot nematodes found in California but susceptible to ring nematodes. *J. regia* is more tolerant of ring but more sensitive to root knot nematode.

Developing resistance to lesion nematode is the most important objective, and evaluation of walnut germplasm has revealed different levels of tolerance of *P. vulnus* in some isolated seedlings, but the majority of the species, including, *J. neotropica*, *J. californica*, *J. ailantifolia*, *J. major*, *J. microcarpa* and *J. nigra*, are seriously affected by this pest (Serr and Day, 1949; Lownsberry *et al.*, 1974). Seedlings that have exhibited some resistance have been propagated for retesting.

Resistance to crown gall. The California walnut industry suffers significant annual losses from crown gall disease in the form of unsaleable nursery stock, lowered productivity of galled trees and increased susceptibility of infected plants to pathogens and adverse environmental conditions (Agrios, 1997). Crown gall is caused by the ubiquitous soil bacterium *Agrobacterium tumefaciens*, and is a chronic problem that affects many fruit, nut and ornamental crops. Although crown gall has been studied for over a century, the continued prevalence of the disease in the field is testament to the limited success of traditional control strategies. Careful cultural practices in nurseries and the use of the biocontrol strain *Agrobacterium radiobacter* K84 are not sufficiently effective.

'Paradox' possesses superior vigour and resistance to abiotic stress, but is more susceptible to crown gall disease (McKenna and Epstein, 2003). The increased prevalence of 'Paradox' rootstock plantings and the abundance of biocontrol (K84)-resistant strains in the field suggest that crown gall disease will

continue to increase as a production problem for the California walnut industry. While some species of walnut exhibit a high level of natural crown gall resistance, traditional breeding approaches could require decades to develop resistance in 'Paradox' rootstock. Recent advances in our understanding of *Agrobacterium* pathology at the molecular level provide a rapid and effective method of reducing the impact of crown gall using interfering RNA transformation (RNAi) silencing (Escobar *et al.*, 2001, 2003). This technology has now been successfully demonstrated in walnut (Escobar *et al.*, 2002). The next step is to apply this method to specific genotypes with additional traits of interest.

1.3.2. Scions

About ten cultivars make up a large proportion of California's orchards. These include 'Hartley', 'Chandler', 'Serr', 'Franquette', 'Payne', 'Vina', 'Ashley', 'Tulare' and 'Howard' (Fig. 10.2.2). Early harvesting cultivars, such as 'Ashley', 'Payne' and 'Serr', are decreasing in hectareage, whereas later cultivars like 'Vina', 'Hartley' and 'Chandler', are stable or increasing. 'Chandler', a University of California release and the latest harvesting of the major cultivars, is also the most extensively planted. This is due to superior attributes of 'Chandler', including mid-season leafing, low incidence of walnut blight and lateral bearing, which contribute to high-quality yield. In addition, its kernel quality is superior, with notably light-coloured kernels

that easily break out into halves. 'Chandler' has set the standard for new cultivars but its popularity combined with its relatively late harvest tends to overload harvesting capacity at the end of the season and may preclude shipments overseas before the holiday season. 'Chandler', like all other English walnuts, is susceptible to blackline disease caused by CLRV and moderately susceptible to codling moth and navel orangeworm.

The ideal walnut cultivar would be relatively late leafing to escape the rains, which spread walnut blight (*Xanthomonas arboricola* pv. *juglandis*), precocious bearing (yielding more than 500 lb/acre in the fourth year) and vegetatively vigorous, with bearing on both terminal and lateral shoots. It would have a low incidence of pistillate flower abscission and other drops and would not be alternate bearing. It would have high production capacity with low chemical input required. The harvest season would end in early October. The nutshell would be relatively smooth, well sealed and make up no more than 50% of the nut weight. The nuts would fit the category of large or jumbo. The kernel would be plump and light coloured, weigh about 7–8 g and come out easily in halves. The tree would be resistant to pests and diseases.

Major breeding objectives. The major breeding objectives are to increase yield, quality and range of harvest dates while decreasing the amount of chemical input required to control pests and diseases.

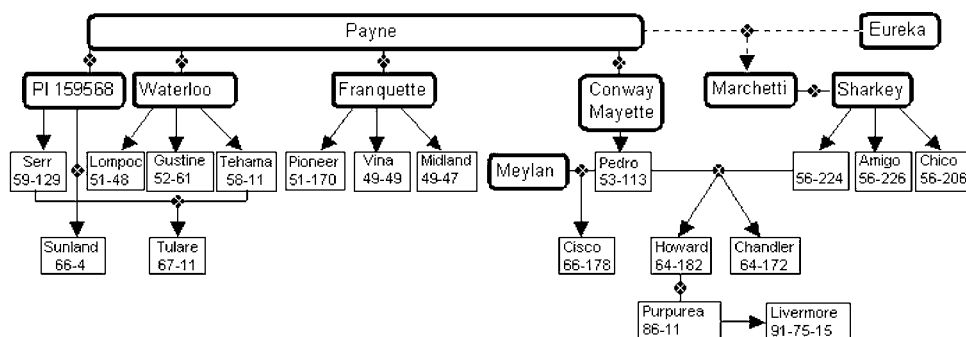


Fig. 10.2.2. Pedigree of major California walnut cultivars and advanced selections in the breeding programme at the University of California, Davis.

Breeding accomplishments. Prior to the Serr–Forde breeding programme (1948–1978), most cultivars grown in northern California, where the industry now resides, were cultivars brought from France by Felix Gillet in the late 1800s or chance seedlings. Gene Serr and Harold Forde made remarkable progress in breeding new cultivars that revolutionized the industry. Their primary breeding objectives were to combine the late leafing and quality of the French types with the lateral fruitfulness and precocity of ‘Payne’ (Fig. 10.2.2). They made 196 crosses, evaluated about 6000 progeny and released 13 cultivars, ten in 1968 and three in 1978. The most important of these are ‘Vina’, ‘Serr’, ‘Howard’ and ‘Chandler’ (Fig. 10.2.2). In 1993, ‘Tulare’ was released from a cross that was made 27 years earlier by Serr and Forde (Fig. 10.2.2; McGranahan *et al.*, 1992). The current breeding programme was initiated in 1983. A walnut cultivar that produces kernels with a red seed coat has recently been released (McGranahan *et al.*, 2001). It is expected that this cultivar will serve a niche market.

Yield. Walnut yield is governed by the number of pistillate flowers produced, the percent set and the size of the nuts. Although tree size may be considered to be a fourth factor, many small trees can produce as many nuts as a few large trees per unit area. The number of pistillate flowers produced is determined by the number of buds that grow each year and the number of shoots that produce flowers. Some cultivars, such as ‘Franquette’, produce flowers only on shoots from subterminal and terminal buds. On other cultivars, e.g. ‘Payne’ and those released from the breeding programme, 80 to 100% of the lateral buds produce pistillate flowers. Cultivars with a high percentage of lateral fruiting are also more precocious, i.e. they come into bearing at an earlier age than terminally bearing types.

Fruit set is determined by pollen availability. Too little pollen results in the drop of unfertilized nutlets at about the size of a marble. This drop is sometimes referred to as ‘June drop’. Another drop, called pistillate flower abscission or PFA, is due to excess pollen and occurs shortly after flowering

(McGranahan *et al.*, 1994b). PFA was first noted on ‘Serr’, where flower drop as high as 80% could occur (Catlin *et al.*, 1987). Removal of pollenizers and mechanical shaking to remove catkins have helped solve the problem, but there appears to be variation in sensitivity to excess pollen and it is important that no more cultivars with a tendency to PFA be released. Occasionally, later drops occur in the growing season and do not appear to be associated with any pest or disease. These drops occur irregularly and have not been extensively studied.

Nut size varies considerably among cultivars and kernel size is reduced as a tree ages (McGranahan and Forde, 1985) and yield increases. The percentage of the nut consisting of the kernel also decreases but the overall weight of the nut remains the same. Current cultivars have about a 6 g nut. New selections have nuts between 7 and 9 g, making up about 50% of the total nut weight. Cultivars with enormous nuts, known as bijou walnuts, usually have a low percentage kernel and low yields and are not used in breeding.

Quality. Quality starts with a well-sealed, relatively smooth, strong shell. The shell must be able to withstand harvesting procedures, which can include drops from > 15 ft. The quality of the shell appears to be genetically determined and shells vary from paper-thin to thick and hard, typical of some of the wild types. Shell strength, integrity and thickness are evaluated in the current programme, and it is more likely to find progeny with shells too thin rather than too thick.

The kernel quality is both genetically and environmentally determined. Commercial walnut meats are graded into two categories: sound (edible) and off-grade (inedible). The sound kernels are separated into grades based on a standard colour chart (Dried Fruit Association, Santa Clara, California). Light grades are the most desirable. All selections in the breeding programme have 100% lights in most years when grown under conditions at the University of California, Davis. We are finding that the same selections may be much darker when grown north near Chico or south near Fresno. Other attributes of the

kernel that are evaluated include amount of packing tissue, kernel plumpness, presence of veins, and shrivel. For descriptors see McGranahan *et al.* (1994a).

Diseases. One of the most important diseases of scions is blight caused by the bacterium *X. campestris* pv. *juglandis* (Teviotdale *et al.*, 2002). Blight is most severe on cultivars that leaf out early (mid-March) and thus are exposed to more of the early spring rains in California. For this reason, a major emphasis in breeding has been mid-season or late leafing cultivars. A late leafing date continues to be the best protection against the disease apart from chemical controls. There have been several claims over the years of blight-resistant selections; however, none of these have proved to be blight-free in California. Differences in susceptibility and response can be noted (Woeste *et al.*, 1992), e.g. 'Serr' will have fewer lesions than many other cultivars that leaf out at the same time.

Blackline, caused by CLRV, is the other major disease of scions, although it is often considered a disease of the rootstock (see rootstocks). Persian walnuts can be systemically infected and show no symptoms, whereas other walnut species exhibit the hypersensitive response. Since the black species are often used as rootstocks, grafted scions are killed when the pollen-borne virus reaches the graft union (Mircetich *et al.*, 1980). We have used back-crossing after discovering that hypersensitivity is governed by a single dominant gene. A hybrid between *J. hindsii* and *J. regia* was used as starting material and the recurrent parents were different *J. regia* cultivars. Progeny of each generation are screened for response to the virus and they segregate 1:1. Only the hypersensitive individuals are carried forward to the next generation. We have now reached the fourth back-cross generation and have progeny that are hypersensitive and closely resemble the Persian parent. Unique to the progeny of the back-crosses is complete male sterility and it is surmised that these may be good populations to use in developing genetically transformed cultivars in which one does not want release of pollen.

Insect pests. Several insect pests attack walnuts. Codling moth (*Cydia pomonella*) is the most damaging because the attacked nuts are unsaleable and serve as a reservoir for the navel orangeworm (*Amyelois transitella*), which is attracted to injured and mummified nuts. The navel orangeworm then does its damage at hull split. As early as 1935, differences in cultivar susceptibility were noted, with phenologically early cultivars like 'Payne' and 'Concord' being more infested than late varieties like 'Franquette'. This is probably due to the timing of codling moth flights with nutlet size and maturity. As a result, late leafing varieties have been a primary goal of walnut breeding since 1968; however, late walnut varieties are attacked if other hosts are not available, and over time codling moth flight timing could adjust to host availability. Control for codling moth is largely insecticide-based, but less ecologically invasive control measures, such as mating disruption and parasitoid release, appear promising. To supplement these control measures we have developed trees expressing the *cryIA(c)* insecticidal protein (*Bt* gene) from *Bacillus thuringiensis*. Laboratory tests have confirmed that high-expressing genotypes are lethal to the codling moth larvae and field trials are under way (Vail *et al.*, 1991; Dandekar *et al.*, 1992, 1994, 1998; Leslie *et al.*, 2001).

Phenology. Phenological traits are highly heritable (0.85–0.96) and are positively correlated (Hansche *et al.*, 1972), suggesting that obtaining a late leafing, early harvesting cultivar would be extremely difficult. In order to produce early harvesting cultivars, the whole growing season must be moved earlier. The solution to this dilemma lies in selecting cultivars for specific growing regions. For example, mid-season cultivars are being released for northern California, where blight is a serious problem; whereas early season cultivars are being released for southern regions that do not have serious problems with blight. Shorter season walnuts have been identified but size appears to be insufficient for release.

2. Molecular Genetics

Molecular genetics encompasses a broad suite of technologies for analysis of genes and their expressed products. Unfortunately, research at the molecular level in walnut has lagged, in large part due to the time and effort it takes to generate molecular information. This is now changing as new and more profound methods are available to investigate the ‘gene space’ of crops like walnut. Genomic approaches that involve a non-biased data collection of genetic information are now available to the scientific community (Bent, 2000; Weinstein, 2002). Robotics is simplifying the analysis of thousands of genes, with the genetic data being analysed by specific computer programs and the useful data stored in public databases. These tools will dramatically improve the availability of genetic information about genes in crops like walnut in the near future.

2.1. Gene cloning

Gene cloning is important for walnut because it is likely to contain many unique genes that may not be discovered in other plants; however, little work with walnuts has been

reported mainly due to the few researchers and the availability of resources to fund this type of work. One of the useful sources of information on cloned walnut genes is GenBank (National Center for Biotechnology Information (NCBI)), the public repository for DNA sequences. Most of the entries are gene sequences. Table 10.2.1 lists GenBank entries by walnut species to provide some idea of the genetic information available for walnut. A significant number of entries correspond to intergenic regions of chloroplast and ribosomal genes. These sequences have been used to investigate the evolutionary relationships among *Juglans* (Potter *et al.*, 2002b; Soltis *et al.*, 2003). Genes specific to walnut that are currently being studied include those involved in tannin, naphthoquinone, unsaturated fatty acid and flavonoid biosynthesis. Several of the genes involved in the biosynthetic pathway of flavonoids have been identified (Beritogoli *et al.*, 2002).

A key determinant of walnut kernel quality is the oil content, about 90% of which is polyunsaturated, and of that 25% is the omega 3-fatty acid α -linolenic acid (ALA) (Greve *et al.*, 1992). The presence of unsaturated fatty acids is an important factor in rancidification of walnuts, in which these acids are oxidized, reducing the shelf-life of

Table 10.2.1. Walnut DNA sequence entries in GenBank.

Species	Intergenic regions		Nuclear encoded genes	Total entries
	Chloroplast genes	Ribosomal genes		
<i>Juglans ailantifolia</i>	1	1	0	2
<i>Juglans australis</i>	1	1	0	2
<i>Juglans boliviana</i>	1	1	0	2
<i>Juglans californica</i>	21	4	0	25
<i>Juglans cathayensis</i>	3	2	0	5
<i>Juglans cinerea</i>	3	2	0	5
<i>Juglans guatemalensis</i>	1	1	0	2
<i>Juglans hindsii</i>	30	5	0	35
<i>Juglans major</i>	15	6	0	21
<i>Juglans mandshurica</i>	4	1	0	5
<i>Juglans microcarpa</i>	18	6	0	24
<i>Juglans neotropica</i>	1	1	0	2
<i>Juglans nigra</i>	18	10	7	35
<i>Juglans nigra</i> $\times$ <i>Juglans regia</i>	0	0	12	12
<i>Juglans olanchana</i>	1	1	0	2
<i>Juglans regia</i>	7	5	951	963

walnut kernels (Greve *et al.*, 1992). The omega-3 fatty acids have been shown to play an important role in growth and development, nutrition and disease prevention. Nutritional studies have demonstrated that walnut consumption can reduce the incidence of coronary heart disease. The genes encoding the various fatty acid desaturases involved in the synthesis of polyunsaturated fatty acids, including fad 2 and fad 3, have been cloned from walnut (A. Dandekar, unpublished data).

2.2. Marker-assisted selection

Marker-assisted selection of progeny in a breeding programme can be greatly facilitated with the use of molecular markers. Certainly, the cloned genes discussed above are excellent markers as long as they display some polymorphism. In addition, molecular markers can be utilized for more traditional genetic strategies utilizing linkage mapping and map-based cloning. Molecular markers have improved the efficiency of linkage mapping, allowing identification of discrete DNA segments where genes of interest reside (Camilleri *et al.*, 1998). Some mapping efforts are ongoing in black (Woeste *et al.*, 2002) and English walnuts (Aradhya *et al.*, 2001). These mapping efforts utilize amplified fragment length polymorphism (AFLP) and microsatellite markers. Microsatellite loci are being used to fingerprint walnut cultivars and, most recently, inter-simple sequence repeat (ISSR) markers have been successfully used to determine the genetic relationships of walnut cultivars (Potter *et al.*, 2002a). Marker-assisted selection is currently in use to identify individuals resistant to CLRV among a back-cross population of English $\times$ black walnut (Woeste *et al.*, 1996a,b).

2.3. Functional genomics

Enzymes in the phenylpropanoid pathway from phenylalanine lead to the biosynthesis of a range of natural products, including the flavonoids. Genes for these enzymes, includ-

ing the key enzyme chalcone synthase, have been functionally investigated in walnuts. Walnuts expressing antisense chalcone synthase were found to be deficient in the accumulation of flavonoids but, interestingly, these deficient plants showed an increase in adventitious root formation (El Euch *et al.*, 1998). These results contrast with other root initiation studies using walnut cotyledons, in which adventitious rooting was observed to occur when the appearance of the lateral root primordia coincided with the expression of chalcone synthase at the same location (Ermel *et al.*, 2000). The genes in the phenylpropanoid and flavonoid pathways were also studied to understand the accumulation of flavonols during heartwood formation in black walnut (Beritogoli *et al.*, 2002). The authors concluded that flavonol synthesis was due to the increased transcriptional activity of genes in the phenylpropanoid pathway in black walnut sapwood cells that are undergoing the transition to heartwood (Beritogoli *et al.*, 2002).

Naphthoquinone metabolism has also been investigated and proteins involved in some of the steps have been identified. Naphthoquinones are important for plant defence and may also be involved in developmental processes (Duroux *et al.*, 1998).

Oil biosynthesis in the embryo is a major metabolic pathway and some effort has been directed at functional characterization of two key steps in the biosynthesis of polyunsaturated fatty acids. Transgenic walnut embryos expressing antisense fad 2 or sense fad 3 have been developed (A. Dandekar, unpublished data) and some of the lines show alterations in the profile and composition of fatty acids. The general idea is that expression of antisense fad 2 will suppress the interconversion of oleic to linoleic acid thus leading to an increase in the accumulation of the monosaturated oleic acid. The expression of sense fad 3 is aimed at over-expressing the enzyme involved in the conversion of linoleic acid to the omega-3 fatty acid linolenic acid. These studies will be useful in developing walnut lines with a high oleic acid (monounsaturated fatty acid) content, as these will be very stable, and also walnuts with an increase in the omega-3 fatty acid.

3. Micropropagation

Walnuts have traditionally been propagated by grafting on to seedling rootstock. Micropropagation has been investigated for propagation of cultivars on their own roots, for production of selected rootstock clones and for development of genetically engineered plants. Walnuts are micropropagated commercially in only one laboratory in Spain (López, 2001). The first reports of walnut micropropagation are from the early 1980s (Chalupa, 1981; Rodriguez, 1982a,b; Cossio and Minolta, 1983; Driver and Kuniyuki, 1984). Micropropagation of mature cultivars (McGranahan *et al.*, 1988a) and better techniques for rooting and acclimatization (Jay-Allemand *et al.*, 1992; Ripetti *et al.*, 1994; Navatel and Bourrain, 2001; Vahdati *et al.*, 2004) have been described more recently. The techniques and further developments have been reviewed (McGranahan *et al.*, 1987; Preece *et al.*, 1989; Leslie and McGranahan, 1992).

Walnut cultures are initiated from disinfested nodal segments of vigorous field- or greenhouse-grown shoots. Driver and Kuniyuki (1984) DKW medium was developed specifically for walnut but success has also been obtained on Murashige and Skoog (1962) (MS) medium. Multiplication occurs through axillary shoot proliferation. Rapid transfer (two to five times per week) is essential after explanting until discoloration of the medium is no longer evident. Once established, cultures need relatively frequent transfer (twice a month) for optimum growth.

Techniques for rooting are still under investigation, and rooting ability is clone-specific. The most promising rooting technique to date utilizes a two-phase system developed by Jay-Allemand *et al.* (1992) and modified by Navatel and Bourrain (2001). Several further modifications have been added (Vahdati *et al.*, 2004). Roots are induced from shoots on medium containing auxin for 3–7 days in the dark. Shoots are then transferred to a root development medium consisting of a mix of quarter-strength basal DKW medium and vermiculite in Mason jars and maintained in the light for 3–4 weeks until roots are visible. Rooted shoots are planted in a well-drained

potting soil and are acclimatized in a fog chamber for 2 weeks, followed by 1 or more weeks on a shaded greenhouse bench. If the terminal buds set, 25 ml/l Promalin® (containing 1.8% GA_{4/7} and 1.8% benzyladenine) can be applied as a foliar spray to stimulate growth.

4. Somatic Cell Genetics

4.1. Regeneration

The presence of tannins, polyphenols and hydroquinone compounds in most vegetative tissues makes the isolation of single cells and protoplasts extremely difficult and challenging.

4.1.1. Somatic embryogenesis

Induction. Development of embryos from somatic tissues has been a very useful tool in the genetic improvement of walnuts. The embryogenic pathway has been used to generate triploids (Tulecke and McGranahan, 1988), intergeneric hybrids (McGranahan *et al.*, 1986) and genetically transformed clones (McGranahan *et al.*, 1988b, 1990a; Dandekar *et al.*, 1989). The techniques were developed for *J. regia* (Tulecke and McGranahan, 1985), but have been applied to other species (Neuman *et al.*, 1993). Immature cotyledonary explants harvested from developing nuts, cultured on conditioning medium for 2–4 weeks and then placed on basal DKW medium, are used for induction of small white somatic embryos from single cells (Polito *et al.*, 1989) on the explants after 8–16 weeks. For induction and maintenance, the cultures are maintained at room temperature in the dark.

Maintenance and development. The somatic embryos are repetitively embryogenic and with monthly subculturing cultures can be maintained for several years. In the light, embryos turn green and a certain percentage will germinate. Germination frequency can be increased following desiccation over a saturated salt solution (Zn₂SO₄, NH₄NH₂, MgCl₂) until embryos have the consistency of popcorn, but not until

browned. Embryos are then returned to DKW basal medium to germinate.

A major challenge in walnut cell culture is to define embryogenic or organogenic cultures from maternal tissue. Efforts to generate somatic embryos from nucellus were unsuccessful (Aly *et al.*, 1992). Repetitively embryogenic cultures were obtained from immature anther tissue of 'Chandler' (G.H. McGranahan, unpublished data), but efforts to replicate this with other cultivars have not been successful. For additional details of methods and progress in walnut embryogenesis, see the reviews by Preece *et al.* (1995) and Tulecke *et al.* (1995).

4.1.2. Recovery of triploids

Walnut endosperm has been used to generate triploids ($3n = 48$) via the embryogenic pathway (Tulecke and McGranahan, 1988). Endosperm was cultured 4–12 weeks post-pollination using standard techniques. The cultivars 'Payne', 'Early Ehrhardt' and 'Manregian' produced repetitively embryogenic cultures. Triploids from endosperm of 'Manregian' are maintained in the *Juglans* germplasm collection at the University of California, Davis. Trees flower and set nuts but embryos do not develop so that the shells are empty and very small.

4.2. Genetic manipulation

Somaclonal variants of walnut have not been identified. A tetraploid walnut, 'Mitsuru', derived by colchicine treatments, has been analysed and compared to the diploid 'Mitsuru' and analysed for pollen characteristics (size, germination rate, fertility, etc.) (Yajima *et al.*, 2003). 'Mitsuru' is known as a Shinano cultivar and was derived from a cross between *J. regia* var. *orientis* Kitamura (Teuchi walnut) and the Persian walnut (*J. regia* L.). Shoots of 'Mitsuru' were exposed to a 0.4% colchicine solution containing 1 ppm of naphthaleneacetic acid (NAA) to produce the tetraploid, which was confirmed as tetraploid by chromosome analysis of shoot tips of the seedlings obtained from open- and self-pollinated plants (Yajima *et al.*, 1997).

4.2.1. Genetic transformation

Walnuts are quite susceptible to *A. tumefaciens* and were one of the first woody plants to be transformed and express foreign genes (Dandekar *et al.*, 1988; McGranahan *et al.*, 1988b).

Breeding objectives. Plant transformation is useful for recalcitrant problems in walnut improvement, including resistance to diseases and pests. Codling moth is the key insect pest of walnut but, because there is little genetic resistance in walnut germplasm that can be utilized, chemical application is the main method for controlling this insect. Crown gall is a serious problem greatly diminishing productivity, and walnuts are very susceptible to this disease. Losses are incurred from both contaminated nursery stock and infected orchard trees. Current prophylactic measures and mechanical removal of galls have not adequately controlled the problem.

Protocol. Walnut is transformed by inserting genes into proliferating somatic embryo cultures. *A. tumefaciens* readily infects young proliferating somatic embryos (McGranahan *et al.*, 1988b). Since new somatic embryos develop from single epidermal cells (Polito *et al.*, 1989), transformed cells produce entirely transformed embryos and chimeras are eliminated. Several independent transgenic lines can be obtained from a single embryo, indicating multiple infection sites on the surface of the walnut embryo (McGranahan *et al.*, 1990a). This feature makes the walnut transformation system very efficient. A detailed protocol for the transformation of walnut has been published (Dandekar *et al.*, 1989). This method was used to produce trees that were transferred to the field in 1989, and these were the first transformed woody fruit or nut tree to be field-tested. These trees bore nuts and the introduced genes were found to be both stably incorporated and inherited in a simple Mendelian fashion.

Accomplishments. A major accomplishment has been the development of walnut

trees expressing resistance to codling moth. Various insecticidal crystal proteins (ICP) from *Bacillus thuringiensis* were tested by incorporating the protein into insect diet (Vail *et al.*, 1991). The *cryIA(c)* protein was found to be the most effective; however, transformation of walnut using the bacterial gene encoding this protein was unsuccessful due to lack of expression (Dandekar *et al.*, 1994). This was a result of the codon bias of the gene sequences taken from *B. thuringiensis* (Dandekar *et al.*, 1994). A synthetic gene correcting this problem worked very well and high levels of codling moth mortality were observed when larvae were fed transgenic embryos (Dandekar *et al.*, 1998). Transgenic trees from these lines are currently being tested in the field.

Recently, we developed a gene silencing strategy to produce resistance to crown gall disease, demonstrating for the first time the use of gene silencing to generate resistance to a major bacterial disease (Escobar *et al.*, 2001). We then applied this approach to walnuts (Escobar *et al.*, 2002). Transgenic walnuts were highly resistant to galling. Resistant genotypes display an absence of macroscopic and microscopic indications of tumorigenesis to a very broad range of *A. tumefaciens* strains, indicating a broad-spectrum durable resistance (Escobar *et al.*, 2003). These plants also provide a unique resource for examining fundamental questions about *Agrobacterium* biology and post-transcriptional gene silencing (PTGS) (Escobar *et al.*, 2003).

Success has also been achieved in modifying tree architecture using the *rolABC* genes from *Agrobacterium rhizogenes*. Transgenic walnut trees expressing these genes show leaf curling, a marked reduction in internode length and altered root architecture, but no increase in rootability (Vahdati *et al.*, 2002).

4.3. Cryopreservation

Zygotic embryos, somatic embryos and pollen have been successfully stored in liq-

uid nitrogen. Walnut pollen with < 7.5% moisture content survives cryostorage for at least 1 year (Luza and Polito, 1988). Satisfactory moisture status is obtained by air drying pollen for 24h after anthesis. Zygotic embryo axes of *J. regia* survive liquid nitrogen storage after desiccation to 5–10% moisture content (Pence, 1990). Somatic embryos survive when treated with 0.2 M sucrose for 24 h and then desiccated to 30–40% moisture before plunging into liquid nitrogen (Setka, 1994).

5. Conclusions

A combination of conventional, *in vitro* and molecular approaches has facilitated considerable progress in walnut improvement. Traditional crossing has contributed to the development of scion cultivars with improved yield, quality, harvest timing and virus resistance and to the development of rootstocks selected for vigour, virus tolerance and nematode resistance. Selection among these crosses has been made more efficient by the development of molecular markers for blackline resistance, cultivar identification and germplasm diversity. Micropropagation and somatic embryogenesis techniques have enabled the development of improved rootstocks and the implementation of genetic transformation. The latter has already been used to develop walnuts resistant to codling moth, the key insect pest, and crown gall disease, a widespread and commercially important rootstock problem. Functional genomics is now beginning to give us a clearer understanding of walnut metabolism and physiology, presenting additional opportunities to improve wood and kernel traits in the near future.

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11

Lauraceae

The *Lauraceae*, together with the *Annonaceae*, *Magnoliaceae* and *Proteaceae*, are considered to be the most ancient angiosperm families. The family itself is thought to have originated from within the *Magnoliaceae* (Scora *et al.*, 2002). Primarily a family of trees, there are only a few economically important species within the *Lauraceae*, and these include spices (bay leaf, *Laurus nobilis*, and cinnamon, *Cinnamomum zeylanicum*), camphor (*Cinnamomum camphora*), timber trees (*Nectandra* spp., *Ocotea* spp. and *Phoebe* spp.) and ornamental trees, e.g. *Persea indica*. The genus *Persea* (Clus.) Miller probably originated in African Gondwanaland, whence it spread to Asia and to North America via Europe; it spread to South America via Antarctica by the Palaeocene, and

the genus was reunited when the land bridge between North and South America was established during the late Neocene. Kostermans (1952) suggested that the South-east Asian *Machilus* Nees, *Nothaphoebe* Blume and *Alseodaphne* Nees are congeneric with *Persea*. Extensive speciation has occurred in Central America (Scora and Bergh, 1990). According to Kopp (1966), there are two subgenera within the genus *Persea*, subgenera *Eriodaphne* (South America) and *Persea* (Central America). There are three species in subgenus *Persea*: *P. schiedeana* Nees, *P. parviflora* Williams and *P. americana* Mill. (Scora *et al.*, 2002). The avocado, *Persea americana* Mill., is the only commercially important fruit species within the family.

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11.1 *Persea americana* Avocado

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1. Introduction

1.1. Botany and history

The humid tropical highlands of Central America, including southern Mexico (Chiapas), Guatemala and Honduras, are considered to be the centre of origin of *P. americana*, and wild *P. americana* trees occur in the forests of this region (Kopp, 1966). There are eight well-defined subspecies or geographical ecotypes of *P. americana* and they appear to have evolved in different climatic environments and in geographical isolation from each other (Scora *et al.*, 2002). These include *P. americana* var. *nubigena* (Williams) Kopp, var. *steyermarkii* Allen, var. *zentmyerii* Schieber and Bergh, var. *floccosa* Mez. and var. *tolimanensis* Zentmyer and Schieber. The remaining three subspecies comprise the common avocado (Bergh and Ellstrand, 1986): (i) the Mexican subspecies *P. americana* var. *drymifolia* (Schlect. and Cham.) Blake, which is thought to have originated in the highlands of south-central Mexico and is adapted to the tropical highlands; (ii) the Guatemalan subspecies *P. americana* var. *guatemalensis* Williams, which is adapted to medium elevations in the tropics; and (iii) the West Indian subspecies *P. americana* var. *americana* Mill., which is

adapted to the lowland, humid tropics (Popenoe, 1941). The Guatemalan race may have originated in the interior valleys of the Central American highlands and its ancestry may include var. *nubigena*, var. *steyermarkii*, var. *zentmyerii* and var. *tolimanensis* (Kopp, 1966; Schieber and Bergh, 1987; Furnier *et al.*, 1990). Williams (1976, 1977) argued that the West Indian subspecies may have evolved from the Mexican subspecies, and Storey *et al.* (1986) speculated that the West Indian avocado may have originated in the Pacific lowlands.

The avocado tree is evergreen, approx. 20 m at maturity. The tree is supported primarily by a shallow unsuberized secondary root system, although anchorage roots can penetrate to 3–4 m (Whiley, 1992). Flowers are borne on the terminals of twigs as panicles of cymes. Avocado fruit is a large, fleshy pyriform or globose berry with a single seed.

The word avocado is derived from the Nahuatl *ahuacatl*, which was transliterated into Spanish as aguacate. The Mexican avocado was apparently selected as early as 7000–8000 BC (Smith, 1966, 1969). According to Gama (1994), domestication of the common avocado most probably occurred over a long period of time among meso-American cultures.

1.2. Importance

World production of avocados was estimated to be approx. 3,040,496 t in 2000 (FAOSTAT, 2004). The leading producing countries include Mexico (1,040,390 t), USA (200,000 t), Indonesia (157,500 t), Brazil (173,000 t), Dominican Republic (150,000 t), and Colombia (144,000 t). Although the avocado is consumed primarily as a fresh fruit, it is also a rich source of oil. The international trade in fresh avocado fruit is very important; the estimated value of avocado exports in 1999 was US\$360,991,000 (FAOSTAT, 2001). Leading exporting countries include Chile, Mexico, Israel, USA and South Africa. The European Union and North America are the biggest importers of avocados. The most important export avocado cultivar is 'Hass'.

1.3. Breeding and genetics

The avocado tree is a large spreading canopy tree in its original habitat. The species is genetically very heterozygous with a long juvenile period and a very high rate of flower abscission and immature fruit drop. Flowering in avocado is dichogamous, protogynous and synchronous (Whiley, 1992). Each flower opens twice during 2 consecutive days. Therefore, all open flowers are either functionally male or functionally female. Cultivars are classified as either type A or type B, depending on whether the flowers are female in the morning (type A) or in the afternoon (type B). This flowering behaviour ensures that self-pollination cannot occur. The chromosome number is $2n = 2x = 24$ (Garcia, 1975). Despite the relatively high costs of conventional breeding of avocado with respect to time, labour and land, conventional breeding programmes have been moderately successful, although leading cultivars are still mostly derived from open pollinations. Bergh and Lahav (1996) and Lahav and Lavi (2002) have reviewed the current status of avocado breeding.

There are no genetic barriers among the avocado subspecies/races, and many cultivars are hybrids involving two or more subspecies. The widely grown 'Hass' and 'Fuerte' avocados are considered to be Guatemalan $\times$ Mexican hybrids. The earliest California cultivars originated either as selections made in Mexico and Central America or from imported seeds (Bergh, 1957). 'Benik', 'Itzamna' and 'Nabal' (Guatemalan) were introduced as bud wood, and 'Dickinson' and 'Fuerte' originated from seed. Openly pollinated seedlings in California were selected later, e.g. 'Bacon', 'Hass', 'Pinkerton', 'Reed' and 'Zutano', and these cultivars are grown in most regions having Mediterranean and subtropical climates. The Florida avocado industry is based upon West Indian and Guatemalan $\times$ West Indian hybrids, and these selections have been successfully introduced to many tropical areas.

1.3.1. Rootstocks

Major breeding objectives.

Phytophthora root rot (PRR). This disease, caused by the soil-borne pathogen *Phytophthora cinnamomi* Rands., is one of the most important limiting factors for avocado production worldwide (Coffey, 1987; Whiley, 1992; Ben-Ya'acov and Michelson, 1995). There is no resistance to PRR in avocado and in other species of subgenus *Persea*. Species within subgenus *Eriodaphne*, however, are highly resistant to the disease, but are sexually and graft-incompatible with species in subgenus *Persea* (Zentmyer, 1980; Bergh and Ellstrand, 1986; Lahav and Lavi, 2002).

Dwarfing. Dwarfing rootstock could have a significant impact on avocado production for several reasons: (i) control of tree size is a major production cost; (ii) harvesting is cheaper from compact trees; and (iii) plantings can be high density, resulting in greater yields.

Salinity stress tolerance. Much avocado production is in areas where water is either saline or in short supply, necessitating the use of brackish water for irrigation, e.g. California, USA, Israel and Australia. Consequently, in order to optimize production, clonal rootstocks should be tolerant of saline conditions.

Breeding accomplishments.

Phytophthora root rot. 'Duke' and its progeny, 'Duke 7', 'Barr-Duke' and 'D9', and 'Thomas' have good tolerance of PRR. The 'G6' selection (Mexican) is also fairly tolerant of PRR. Some of the PRR-tolerant selections have serious limitations. 'Martin Grande' (G755), a cross involving *P. schiedeana* × *P. americana* (Guatemalan race) (Ellstrand *et al.*, 1986), produces low-yielding trees. Tsao *et al.* (1992) observed that some rootstocks with tolerance of *P. cinnamomi* have no tolerance for another serious soil-borne pathogen, *Phytophthora citricola*. Guatemalan rootstocks appear to be more sensitive than Mexican rootstocks to *Dothiorella* and *Verticillium* wilt (Zentmyer *et al.*, 1965).

Dwarfing. Although Sánchez-Colin and Barrientos-Priego (1987) reported that 'Colin V-33', used either as an interstock or as a rootstock, could confer dwarf habit to the scion, this selection has tested positive for the avocado sunblotch viroid (ASBVd).

Soil stress. West Indian avocados demonstrate the greatest resistance to salinity and Mexican cultivars are least resistant, although there is significant variability within each race (Kadman and Ben-Ya'acov, 1976) and among seedlings from the same tree (Kadman, 1968). Resistance to Ca²⁺-induced chlorosis is greatest in West Indian cultivars, but there is also considerable variability for this trait within seedling populations (Ben-Ya'acov, 1972). West Indian rootstocks perform poorly in heavy soils and under waterlogged conditions (Ben-Ya'acov *et al.*, 1974).

1.3.2. Scions

Major breeding objectives.

Fruit quality. The international market standard for avocados is the black-skinned 'Hass' and, to a much lesser extent, the green-skinned 'Fuerte'. Several cultivars that closely resemble 'Hass' have been released in order to supplement this selection, particularly in its off-season, and these include 'Gwen', 'Jim', 'Lamb Hass' and 'Reed'. The optimum fruit size for most markets is about 250–350 g (Lahav and Lavi, 2002). Size is highly variable in each genotype, and is affected by stage of maturity, cultural practices and climatic conditions (Lahav and Kalmar, 1977; Whiley and Schaffer, 1994). The shapes of pyriform 'Hass' and ovate 'Bacon' and 'Gwen' are desirable. The easily removed peel of 'Fuerte' and 'Hass' is preferred. In Mexican and Mexican × Guatemalan types, small seed size is desirable; however, this trait is uncommon in West Indian avocados.

Shelf-life. Mexican-type avocado fruit are strongly climacteric (Adato and Gazit, 1977). According to Eaks (1980), a fixed climacteric phase appears to precede a variable lag phase. Low levels of endogenous ethylene accumulate during the lag phase and trigger the climacteric as sensitivity to ethylene increases (McMurchie *et al.*, 1972). Following its initiation, ripening cannot be arrested. According to Whiley (1992), fruit of Guatemalan and Mexican cultivars can be stored on the tree during the lag phase for 2–4 months after reaching maturity, and accumulate oil during this period. Ripening begins to occur only after the fruit are picked. West Indian and West Indian × Guatemalan cultivars, on the other hand, cannot be stored in this manner, and must be picked at maturity. On-tree storage together with different climatic conditions has enabled producers in many areas to concentrate production on a single cultivar, 'Hass' (Griswold, 1945). In order to assure year-round production of West Indian and West Indian × Guatemalan fruit in the tropics, several cultivars having different maturity

dates must be grown (Crane *et al.*, 1996). Consequently, there is no single market standard for tropical avocados.

Fruit diseases. In the humid tropics and subtropics, fruit diseases are a serious problem. 'Fuerte' and other Mexican avocados are highly susceptible to anthracnose or black spot (*Colletotrichum gloeosporioides* Penz.) (Ruehle, 1963). 'Collinson', 'Fuchsia' and 'Pollock' are apparently fairly resistant to cercospora spot or blotch (*Pseudocercospora purpurea* Cooke). 'Fuchsia', 'Pollock', 'Booth 1' and 'Waldin' are moderately resistant to avocado scab (*Sphaceloma persea* Jenkins).

Tree architecture. Compactness and dwarf or semi-dwarf trees are ideal for ease of harvesting, grove management and high-density plantings. According to Lahav and Lavi (2002), tree vigour and fruitfulness are inversely related, and robust seedling trees often have few fruit.

Breeding accomplishments. Classical breeding has provided relatively little genetic information about *Persea*. With respect to Mendelian genetics, the dwarfing character of *P. schiedeana* has been attributed to a single gene (Bergh and Lahav, 1996). Lavi *et al.* (1993a,b) have concluded that morphological traits in avocado are probably coded by several loci with several alleles in each locus. Several cultivars have been released from the California and Israeli avocado breeding programmes.

2. Molecular genetics

Two main avenues of research involving molecular studies have been followed: the generation and application of various markers and the identification of avocado genes and expressed sequence tags (ESTs).

2.1. Gene cloning

Cellulase was the first gene to be identified in avocado (Christoffersen *et al.*, 1984); pAV5

is a ripening-specific complementary DNA (cDNA) clone identified to be a cellulase gene on the basis of co-migration of the *in vitro* translation product, on SDS-PAGE and immunoprecipitation by antiserum to purified avocado cellulase. This enzyme is regulated at the transcription level since a 50-fold increase in cellulase mRNA occurs during ripening. Tucker *et al.* (1987) used the pAV5 clone to screen a cDNA library prepared from ripe 'Hass' fruit and isolated clone pAV363 containing 2021 transcribed nucleotides. The full-length message is approximately 2.2 kb, having an open reading frame (ORF) of 1482 bp coding for a 54.1 kDa polypeptide. Further analysis of this family was carried out by Cass *et al.* (1990), who screened an avocado genomic library with the cellulase cDNA probe and identified two family members: *cel1* and *cel2*; *cel1* is highly homologous to cellulase cDNA and represents the ripening-related cellulase gene, whereas *cel2*, although related to *cel1*, is divergent in its 5' end and no cellulase cDNA derived from ripe fruit represents *cel2* transcripts. Thus, the *cel1* gene is responsible for the major portion, if not all, of the cellulase transcripts in ripe fruit. Only *cel1* transcripts have been detected in ripe mesocarp and its accumulation has been detected in the fruit abscission zone (Tonutti *et al.*, 1995). It has been suggested that the *cel1* expression in the mesocarp during fruit ripening is associated with abscission of mature avocado fruits. Several control upstream motifs have been identified in the cellulase and other ethylene-regulated genes.

A cytochrome P-450 has been purified from the mesocarp of ripe avocado fruit by O'Keefe *et al.* (1992). The enzyme is functional as *p*-chloro-*N*-methylaniline demethylase. Bozak *et al.* (1990) isolated two overlapping cDNAs that accumulate during ripening and which were identified as P-450 (Bozak *et al.*, 1992). The N terminus of the predicted polypeptide derived from the cDNA is identical to the N terminus of the purified avocado enzyme. The gene was designated as CYP71A1 and the mRNA was determined to be 1800 bp. The predicted protein has less than 40% homology with other genes of the P-450 family and thus

could represent a separate family. During ripening, there is accumulation of the CYP71A1 gene product, which correlates with increased total P-450 and enzyme activities. The functional role of the CYP71A1 gene remains obscure. Substrate studies (Christoffersen *et al.*, 1995) showed that various monoterpenes, e.g. nerol and geraniol, are either hydroxylated or epoxylated by the CYP71A1 enzyme, although these have not been detected in ripening fruit.

Avocado polygalacturonase (PG) cDNA has been isolated from a cDNA library by heterologous hybridization with a tomato cDNA probe. The full-length mRNA is 1900 bp, coding for 453 amino acids, which is similar in size to the reported avocado PG protein (Kanellis *et al.*, 1991). Avocado PG is similar to the tomato and maize enzymes and contains the conserved octapeptide which characterizes PGs from plants, fungi and bacteria (Dopico *et al.*, 1993; Kutsunai *et al.*, 1993). Wakabayashi and Huber (2001) have isolated an endo-PG from cell walls of avocado mesocarp by sequential ion exchange and gel permeation chromatography. They recovered two isoforms of approximately 46 and 48 kDa, while the latter has slightly higher catalytic activity. The purified enzymes catalysed significant molecular mass downshifts in the polyuronides of pre-ripened fruits; however, they had limited capacity to solubilize polyuronides from cell walls of these fruits.

The AVOe3 mRNA has been identified as a ripening-related gene and is detected after 12 h of propylene treatment. The clone encodes a soluble, monomer polypeptide of 34 kDa. Because of its pattern of induction and its relationship to an ethylene-related tomato gene, it might be involved in ethylene biosynthesis (McGarvey *et al.*, 1992).

Twenty-three cDNA clones have been identified by differential screening of a cDNA library made from 7°C stored fruits (Dopico *et al.*, 1993). These clones were grouped as ten families, six of which had increased expression during cold storage and normal ripening. These sequences had homologies to PG, endochitinase, a cysteine proteinase inhibitor and several stress-

related proteins. In clones from six families, no homology was detected.

The ethylene-forming enzyme (EFE) has been cloned and over-expressed in *Escherichia coli*. This Fe (II)- and ascorbate-requiring oxidase is responsible for the last step of ethylene biosynthesis, in which 1-aminocyclopropane-1-carboxylic acid (ACC) is converted to ethylene. The kinetic mechanism of ACC oxidase is currently being investigated (R. Cristoffersen, University of California, Santa Barbara, personal communication).

At the time of writing, there have been 140 avocado sequences, including sequences of ASBVd, in the NCBI database.

2.2. Genetic markers and marker-assisted selection

Molecular tools for marker identification and mapping are increasingly being exploited in avocado breeding and genetic analysis. Isozymes have been used as genetic markers in avocado (Torres and Bergh, 1980); however, current research has focused on the application of various DNA markers, e.g. restriction fragment length polymorphism (RFLP) (Furnier *et al.*, 1990; Bufler and Ben-Ya'acov, 1992), random amplified polymorphic DNA (RAPD) (Bufler and Ben-Ya'acov, 1992) and variable number of tandem repeats (VNTR), including minisatellites (Lavi *et al.*, 1991) and microsatellites or simple sequence repeats (SSRs) (Sharon *et al.*, 1997).

2.2.1. Assessment of heterozygosity

Genetic variation and the level of heterozygosity in avocado have been studied. The level of heterozygosity was found to be 100% in five crosses and 90–94% in the analysis of self-pollinated progeny using mini- and microsatellites (Lavi *et al.*, 1994). Typing of 59 loci with microsatellites in five avocado cultivars revealed an average heterozygosity of 0.58 (Nei and Roychoudhury, 1974). Gene diversity (Rongwen *et al.*, 1995) was 0.42–0.66. Analysis of 11 cultivars with 17 microsatellites revealed an average of 6.1

alleles per locus, a heterozygosity level of 0.79 and an average gene diversity of 0.78. A relatively low level of self-pollination could explain the high level of heterozygosity in avocado.

2.2.2. Genetic relationships

Davis *et al.* (1998) attempted to determine the paternal origins of important avocado cultivars and the frequency of outcrossing in populations of avocados using RFLP and microsatellite markers. Furnier *et al.* (1990) used RFLP markers and suggested that *P. nubigena* and *P. steyermarkii* are ancestral to *P. americana* var. *guatemalensis*, as suggested by Kopp (1966) and Scora and Bergh (1990). In addition, they suggested that *P. americana* consists of *P. schiedeana* and a large taxon containing the other species and varieties. Thus, *P. floccocosa* may be a variety of *P. americana* and the root rot-tolerant rootstock G755A probably resulted from a cross between *P. americana* and *P. schiedeana*. Bufler and Ben-Ya'acov (1992), using ribosomal DNA probes, were able to identify var. *drymifolia*, while *guatemalensis* and *americana* could not be separated. This is in agreement with Kopp (1966), but is in contrast with Scora and Bergh (1990) and Pliego-Alfaro and Bergh (1992), who suggested that the three races are equally distinct from each other. These authors have used RAPDs to distinctly identify each of the avocado races and various avocado accessions.

The relationship among avocado cultivars and among *Persea* species using mini- and microsatellite markers was explored by Mhameed *et al.* (1997), who assigned 19 avocado cultivars (out of 24 tested) to each of the three avocado races. Other cultivars of unknown origin were compared with DNA patterns of DNA mixes and were found to be hybrids between the various races. In addition, the Guatemalan and the West Indian races were found to be more closely related to each other than to the Mexican race. Mhameed *et al.* (1997) observed that the *P. americana* races and three accessions of *P. schiedeana* are quite

distinct from each other. Neither the morphological nor the DNA markers are superior to each other in phylogeny studies and the two tools should complement each other (Scora *et al.*, 2002). Thus, the relatively high levels of genetic variation observed in selfing progeny using morphological and DNA markers questions the validity of races and species in avocado (Mhameed *et al.*, 1997).

2.2.3. Genetic mapping and marker-assisted selection

Fifty progeny of the cross 'Ettinger' × 'Pinkerton' were genotyped by 93 microsatellites (of which 51 were found to be polymorphic in this family), 17 polymorphic RAPD markers and 23 minisatellite markers (Sharon *et al.*, 1997). The resulting preliminary genetic map consists of 12 linkage groups having two to five markers in each group (a total of 35 markers) and covering about 357 cM. Mhameed *et al.* (1995) identified DFP fragments associated with skin colour, harvest duration, skin thickness, skin surface, fruit weight, seed size and peeling ability. The fragment P8 was associated with black-purple skin colour based upon two half-sib families. Sharon *et al.* (1998) also used microsatellites to identify specific bands that are associated with genes controlling skin gloss and seed size. The SSR marker AVAO4 (mapped to linkage group 3) is linked to a gene controlling the amount of fibre in the flesh ($P = 0.00001$). Correlations between morphological and DNA markers together with a linkage map should eventually enable marker-assisted selection for avocado improvement.

3. Micropropagation

The main goal for micropropagating avocados is for clonal rootstocks with tolerance of soil-borne diseases caused by *P. cinnamomi* and *Rosellinia necatrix* and of saline or calcareous soil limestone conditions. Ontogeny of the explants, juvenile or adult, is critical for *in vitro* development of shoots.

3.1. Explanting and shoot proliferation

3.1.1. Juvenile material

Nodal sections with lateral buds have been used successfully for *in vitro* establishment of 1- to 4-year-old seedlings of 'Hass' and 'Hopkins' (Cooper, 1987) and of 1- to 3-year-old seedlings of 'Fuerte' (Schall, 1987) and 'Duke' (Capote *et al.*, 2000). *In vitro*-germinated seedlings of 'Gvar-am 13' have been used as a source for explants (Barceló-Muñoz *et al.*, 1990), while Barringer *et al.* (1996) used embryonic axes as explants for several West Indian cultivars.

Mineral salts and hormonal balance are important for culture initiation and subsequent shoot proliferation. Cooper (1987) recommended the woody plant medium (WPM) formulation (Lloyd and McCown, 1981) with 4.44 μM benzyladenine (BA). To initiate shoot elongation, medium containing 1.3 μM BA and 0.5 μM indolebutyric acid (IBA) was used. Under these conditions, a threefold multiplication rate per month could be obtained over a 2-year period. Barceló-Muñoz *et al.* (1990) utilized the N_{45}K macroelements formulation (Margara, 1984) with 45 mM total N content (in a 4:1 $\text{NO}_3^-/\text{NH}_4^+$ ratio) and 4.44 μM BA. After several subcultures, shoots became miniaturized and could be multiplied for several years. For shoot elongation, 0.3–0.5 cm shoots are cultured in liquid medium for 2 weeks in the presence of 1.3 μM BA or kept in multiplication medium without subculturing for 8–10 weeks. Similarly, Witjaksono *et al.* (1999b) obtained optimum growth on a modified Murashige and Skoog (1962) (MS) formulation with 67% NO_3^- and 33% NH_4^+ and 40 mM total N content.

The effects of irradiance level and medium (semi-solid or double-phase) on shoot proliferation have been studied by de la Viña *et al.* (2001), using shoot cultures derived from 2-year-old seedlings. Cultures on semi-solid medium with 2.89 μM BA resulted in reduced shoot length and proliferation compared to those growing under double-phase conditions (same composition of semi-solid medium but with 3 ml of liquid medium supplemented with 0.44 μM BA

overlying the semi-solid phase). In 30% of cultures growing on semi-solid medium, the main shoot demonstrates symptoms of apical necrosis after 3 subcultures, while axillary shoots growing under double-phase conditions are hyperhydric. Chlorophyll a and carotenoid concentrations generally decrease when irradiance is increased, e.g. shoots growing under 60–85 $\mu\text{mol}/\text{m}^2/\text{s}$ are yellowish while those at 35 $\mu\text{mol}/\text{m}^2/\text{s}$ are green. Scanning electron microscopy studies have shown that leaf stomata from double-phase medium remain open, deformed and asymmetric, while those from semi-solid medium are half open and below the level of epidermal cells. Double-phase conditions negatively affect rooting of cuttings. Barceló-Muñoz (1995) observed that spongy parenchyma cells of hyperhydric shoots are larger and with larger intercellular spaces than those of control leaves; moreover, starch accumulates in the chloroplasts, probably due to their inability to export sugars. Length of hyperhydric shoots is greater and they have a slightly lignified vascular system.

3.1.2. Mature phase material

Schall (1987), working with axillary buds of adult phase 'Fuerte', initiated axillary bud proliferation on semi-solid, half-strength MS medium with 22.2 μM BA. Shoot development occurred on semi-solid medium with 4.44 μM BA; however, shoots could only be maintained on proliferation medium for three to four subcultures. Similar results have been obtained with 'Duke 7' (Harty, 1985; Cooper, 1987). Harty (1985) used semi-solid Dixon and Fuller (1976) (DF) medium with 46 μM kinetin, and Cooper (1987) used WPM, a semi-solid medium, with 0.44 μM BA. Using etiolated axillary buds of 'Duke', Capote *et al.* (2000) obtained bud sprouting and shoot growth on DF medium with 8.88 μM BA and 5.77 μM gibberellic acid (GA_3); however, growth decreased with subculturing.

Using nodal sections with axillary buds from severely pruned IV-8 rootstocks, Pliego-Alfaro *et al.* (1987) obtained proliferating shoots on semi-solid, half-strength MS

medium with 1.3 μM BA. To stimulate proliferation, shoots are maintained in double-phase medium with 2.8 and 0.4 μM BA in the solid and liquid phases, respectively; however, hyperhydricity increases with subculturing. This method has also been used to multiply adult material of 'Duke', 'Fuerte', 'Hass' and 'Topa-Topa' (Zirari and Lionakis, 1994), although data on shoot development have not been presented. To control hyperhydricity, shoots of IV-8 must be cultured for 2 weeks in liquid B5 medium (Gamborg *et al.*, 1968) containing 1.3 μM BA, followed by 6 weeks in double-phase B5 medium with 2.8 and 0.4 μM BA in the semi-solid and liquid phases, respectively. Shoots have been maintained under these conditions for several years (Barceló-Muñoz *et al.*, 1999).

Adult shoots of 'Gvar-am 13' have been maintained as proliferating cultures for > 2 years in N_{45}K macroelement formulation with 4.44 μM BA, although weekly subculture is needed to avoid apical necrosis (Pliego-Alfaro *et al.*, 1987). Hyperhydricity increases with subculturing under these conditions.

For avocado cultivars that are difficult to establish *in vitro*, grafting of axillary buds on to *in vitro*-germinated seedlings (Pliego-Alfaro and Murashige, 1987; Barceló-Muñoz, 1995) can be used to overcome the problem. Shoots of *in vitro*-grown 4-week-old seedlings are decapitated, their axillary buds removed and lateral buds inserted into an incision in the rootstock. Approximately 70% of the micrografts develop successfully, providing shoots after 6–8 weeks. Shoots generally show a rapid decline in growth rate after separation from the rootstock (Barceló-Muñoz, 1995).

3.2. Rooting

Rooting of juvenile shoots can be obtained as long as material of adequate size (> 1.5 cm with several leaves) is used. Auxin and nutrients are not essential for induction, although their presence favours rooting. In the absence of auxin, Pliego-Alfaro (1988) obtained 60% rooting without basal medium, 100% with $0.3 \times \text{MS}$ salts and 10% with full-

strength MS medium. Rooting also occurs in the absence of sucrose. Pliego-Alfaro (1988) found no difference in rooting rate of juvenile shoots in 0 to 6% sucrose, although callus production increased with sucrose concentration and some yellowing of leaves was observed at the 6% level. No differences in root number were found in 3 to 6% sucrose, while in the absence of sucrose root number is reduced by half. Premkumar *et al.* (2003) associated decreased rooting with yellowing of the leaves at 5% sucrose.

Continuous exposure to auxin does not significantly affect root induction; however, short exposures (3 days) to 123–492 μM IBA significantly increase rooting (Pliego-Alfaro, 1988). Barceló-Muñoz *et al.* (1990) showed that 4.92 μM IBA is equally effective. Cooper (1987) dipped shoots for 1 s in 1000–3000 mg/l IBA or naphthaleneacetic acid (NAA), before transfer to basal medium. IBA induces slightly higher rooting percentages, but NAA promotes faster rooting. The physiological stage of the shoots affects rooting, e.g. etiolated shoots generally root poorly and auxin reduces their rooting potential (Pliego-Alfaro, 1988). De la Viña (1996) recommends incubation of light-grown shoots under dark conditions for 3 days with exposure to auxin in order to increase rooting.

Due to the inhibitory role of auxin in root elongation, Pliego-Alfaro (1988) used activated charcoal in the second phase of rooting, while Schall (1987) has recommended 24.6 μM IBA throughout the rooting process, and increased rooting from 26% to 87% after supplementing the medium with activated charcoal.

Auxin metabolism during rooting has been studied by García-Gómez *et al.* (1994). Concentrations of indoleacetic acid (IAA) and the conjugate IAA-aspartate were measured in juvenile shoots that rooted in the absence of auxin and in cuttings treated with IBA or with the auxin transport inhibitor TIBA. They demonstrated the importance of endogenous auxin for rooting, e.g. no rhizogenesis was observed in the presence of TIBA, while activation and development of cambial cells was not inhibited. García-Gómez *et al.* (1994) suggested that auxin is necessary at the early stages of rooting, e.g.

activation of cambial cells (days 0–3) and division of cambial cell derivatives (days 3–6). García-Gómez *et al.* (1995) observed increased peroxidase activity between days 3 and 6 of the rooting process. Histological studies indicated that there is a close association between peroxidase activity and cambial cell division and differentiation.

Ex vitro rooting has been demonstrated with juvenile avocado. Cooper (1987) dipped shoots for 1 s in 3000 mg/l NAA and obtained 100% rooting. Under these conditions shoots formed excellent root systems.

Rooting potential of adult shoots is low and does not increase with subculture (Pliego-Alfaro *et al.*, 1987). Generally, shoot growth ceases, the leaves are shed and the shoots die following transfer to rooting medium (Pliego-Alfaro and Murashige, 1988; Zirari and Leonakis, 1994). Rooting can be improved following grafting of adult buds on to *in vitro*-germinated seedlings. Pliego-Alfaro and Murashige (1988) obtained 50% rooting of adult 'Duke-7' shoots after one graft, although regrafting (as many as three times) did not improve rooting or shoot vigour. Up to 90% rooting of adult 'Gvar-am 13' shoots occurs following successive *in vitro* grafting (as many as 15 times) (Barceló-Muñoz, 1995). The restored rooting competence is stable after nine subcultures but proliferation is poor.

Approximately 30% rooting has been obtained with shoots derived from severely pruned IV-8 plants (Pliego-Alfaro *et al.*, 1987). Rooting increases up to 72% if the root induction phase (with auxin) is in liquid medium, and up to 77% if root induction and elongation phases occur in liquid medium. Unfortunately, in the latter case, virtually all shoots are hyperhydric, and a liquid–solid medium alternation is preferable. Up to 90% rooting can be obtained with B5 medium, demonstrating the important role of medium on rooting (Barceló-Muñoz *et al.*, 1999).

3.3. Acclimatization

The physiology of juvenile avocado plantlets growing under *in vitro* conditions has been studied. For example, de la Viña *et al.* (1999)

investigated the effects of sucrose concentration and carbon dioxide levels on photosynthesis and determined the amount of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco protein) in rooted avocado shoots. At high sucrose levels (87.6 mM), Rubisco content is low in leaves, particularly in the presence of high CO₂ (1000 μmol/mol). Under these conditions, the maximum photosynthetic rate (P_{max}) is low.

In a similar study, Witjaksono *et al.* (1999b) grew avocado plantlets in a CO₂-enriched environment with 30 g/l sucrose, and observed that the net CO₂ assimilation rate was reduced by 39%, although these mixotrophic plantlets grew better than those cultured under atmospheric CO₂ conditions. De la Viña *et al.* (1999) also obtained higher leaf area and leaf fresh weight (FW)/(stem + root) FW ratio in plants grown under enhanced CO₂, although survival after transplanting was less for plants coming from low sucrose/high CO₂ conditions in comparison to those grown under high sucrose/high CO₂.

The effect of sucrose in the culture medium prior to acclimatization of juvenile avocado plantlets has been studied by Premkumar *et al.* (2002, 2003). No significant differences in total leaf chlorophylls, N mass, flavonoids and total soluble proteins were found in plants under *in vitro* or *ex vitro* conditions. However, *ex vitro* transfer strongly affects foliar ratios of chlorophyll a to chlorophyll b and total chlorophylls to carotenoids, indicating that transfer *ex vitro* implies a modification of the light absorption by the leaves. The C : N ratio is affected by changes in sucrose concentration as well as by transfer *ex vitro*, suggesting increased structural carbon that is related to hardening of cell walls and increased xylem tissue (Premkumar *et al.*, 2002). Rubisco content decreases with increasing sucrose content, while it greatly increases *ex vitro*, suggesting a possible role as storage pool of reduced nitrogen for growth *ex vitro* (Premkumar *et al.*, 2002). Increased sucrose does not alter the endogenous levels of monosaccharides, sucrose and starch in leaves *in vitro*. In general, endogenous concentrations of sucrose and starch are higher in leaves than in roots,

irrespective of the sucrose treatment, while the starch content increases in leaves of *ex vitro*-acclimatized plants and sucrose level is lower. These changes indicate improved sucrose utilization efficiency and starch synthesis *ex vitro* (Premkumar *et al.*, 2003).

Acclimatization of rooted juvenile shoots under high relative humidity (RH) can be routinely accomplished (Cooper, 1987; Schall, 1987; Barceló-Muñoz *et al.*, 1990). However, growth and development are improved after inoculation with vesicular-arbuscular mycorrhizal fungi. Vidal *et al.* (1992) obtained improved rooting and shoot growth following inoculation with *Glomus fasciculatum* at the time of transplanting, together with improved survival of inoculated plants. De la Viña *et al.* (1996) obtained similar results with RR-86 rootstock, a 2-year-old seedling showing tolerance of *P. cinnamomi*, after inoculating rooted shoots with *Glomus deserticola*.

Adult plantlets show slow growth *ex vitro*, resulting in a low survival rate (Barceló-Muñoz, 1995). Barceló-Muñoz *et al.* (1999) obtained 70% survival after maintaining IV-8 plants for > 4 weeks in polyethylene tunnels with 100% RH, 110–120 $\mu\text{mol}/\text{m}^2/\text{s}$ irradiance level at 15–30°C. Plants were exposed to increasing periods (5 min the first day, 5 h the last day) of ambient environmental conditions for 4 weeks. After 4 weeks, plants could be transferred to open tunnels.

4. Micrografting for Elimination of Pathogens

ASBVd, a member of the Avsunviroidae family of viroids, causes sunblotch disease of avocado. ASBVd causes symptoms on fruits and leaves, and levels of infection in the plant vary according to the tissue origin and environmental conditions (da Graca and Moon, 1983; Schnell *et al.*, 1997). Asymptomatic trees can occur (Semancik and Szychowsky, 1994) and infected plants have appeared to be healthy after further indexing, which is interpreted as unequal distribution of the viroid, lack of efficiency of the indexing technique and silencing mechanism of the host on the viroid (Olano *et al.*, 2002). Transmission of ASBVd can occur via

seeds, vegetative material, pollen and contaminated tools (Parker and Horne, 1932; Wallace and Drake, 1962; Desjardins *et al.*, 1979, 1987); and the only effective control is eradication of affected plants.

Shoot tips, consisting of the meristem plus two to three leaf primordia, from *in vitro*-germinated avocado seedlings of ASBVd-infected cultivars were micrografted on to decapitated seedlings of two ASBVd-free cultivars, and plants were recovered (Suarez, 2003; Suarez *et al.*, 2004). Regenerated plants were indexed by RT-PCR for ASBVd infection and amplified products were cloned and sequenced. RT-PCR indicated that ASBVd replicated in the micrografts, while no ASBVd was detected in micrografts from plants that tested negative. In a parallel study, Suarez (2003) screened embryogenic cultures and plants derived from avocado nucellus, and demonstrated with RT-PCR that ASBVd was present in all cultures. ASBVd, therefore, cannot be eliminated either by *in vitro* micrografting or nucellar culture (Suarez, 2003).

See micrografting for rejuvenating mature phase avocados in Section 3.12 above.

5. Somatic Cell Genetics

5.1. Regeneration

The chief prerequisite for applying somatic cell genetic approaches to avocado, including protoplast-based technologies, *in vitro* mutagenesis and selection and genetic transformation, is a highly efficient regeneration protocol from single cells of elite selections. There were several early reports of callus initiation from various plant parts of avocado; however, the callus was non-morphogenic (Schroeder, 1956, 1961, 1971, 1980; Blumenfeld and Gazit, 1971).

5.1.1. Somatic embryogenesis

Induction. Conditions for induction of embryogenic cultures from explanted avocado zygotic embryos, using openly pollinated 'Hass' (Mexican $\times$ Guatemalan) as a

model, were described by Pliego-Alfaro (1981) and Pliego-Alfaro and Murashige (1988). These reports have been confirmed by Mooney and van Staden (1987) with openly pollinated 'Fuerte' (Mexican $\times$ Guatemalan) and with 'Duke 7' (Mexican) and later by Witjaksono and Litz (1999a,b) and Raviv *et al.*, 1998. Various sizes and developmental stages of zygotic embryos have been utilized as explants. Mooney and van Staden (1987) used 0.1–0.5 mm embryos from 3–4 mm fruits, Pliego-Alfaro and Murashige (1988) used 0.6–0.8 mm embryos from 9 mm fruits and Raviv *et al.* (1998) used 7–10 mm embryos. After surface sterilization, the fruit are bisected longitudinally. The immature seed halves can be plated on induction medium so that the zygotic embryo is in contact with medium or the zygotic embryo can be excised and used as the explant. Witjaksono *et al.* (1999a) demonstrated that embryogenic cultures could also be induced from the nucellus (maternal) of immature avocado seeds of the same stage of development on the same induction medium. Cultures are induced on semi-solid induction medium in 60 mm $\times$ 15 mm plastic disposable Petri dishes in darkness at 25°C.

Induction of embryogenic cultures from explanted nucellus (Witjaksono *et al.*, 1999a) and zygotic embryos (Pliego-Alfaro, 1981; Pliego-Alfaro and Murashige, 1988; Mooney and van Staden, 1987) has been reported on semi-solid MS medium supplemented with 0.1 mg/l thiamine HCl, 100 mg/l myoinositol, 30 g/l sucrose and 0.41 μ M picloram. Witjaksono and Litz (1999a) observed that an induction basal medium consisting of B5 major salts (without $(\text{NH}_4)_2\text{SO}_4$ with MS minor salts and organic components was superior to MS medium. Embryogenic cultures are induced approx. 8–25 days after explanting. Cultures consist of proembryonic masses (PEMs) and hyperhydric somatic embryos.

Maintenance. Medium-term maintenance of embryogenic cultures is essential for many types of *in vitro* manipulation. Witjaksono and Litz (1999a) optimized the growth of embryogenic cultures on semi-solid MS

induction medium formulation supplemented with agar, but observed that more somatic embryos developed on modified B5 medium (see above) supplemented with gellan gum. Subculture of the smallest PEM fraction at 3–5-week intervals is necessary for cultures on semi-solid medium. Maintenance of embryogenic cultures in suspension, however, provides optimum conditions for proliferation of PEMs (Witjaksono and Litz, 1999a). MS medium that has been modified to contain 12 mg/l NH_4NO_3 and 30.3 mg/l KNO_3 results in the highest PEM FW increase compared to other medium formulations (Witjaksono and Litz, 1999b). Biweekly subcultures are necessary for embryogenic suspension cultures. Usually, 0.5 or 1 g PEMs are inoculated into 40 ml or 80 ml liquid maintenance medium in 125 ml or 250 ml Erlenmeyer flasks, respectively.

There are two distinct types of embryogenic avocado cultures (Witjaksono and Litz, 1999a), and these responses are genotype-dependent:

1. The PEM type. A few genotypes proliferate as PEMs with no differentiation of cotyledons in the presence of auxin.
2. The somatic embryo (SE) type. Heart and later developmental stages of somatic embryos differentiate and develop in the presence of auxin, and this is the most typical response.

Suspension cultures of the SE type must be sieved prior to subculture, and only the <0.8 mm fraction is inoculated into fresh maintenance medium. The loss of embryogenic potential under maintenance conditions is cultivar-dependent (Witjaksono and Litz, 1999a) and can vary from 3 months with 'Yon' to >2 years with 'Esther'. Cultures on semi-solid maintenance medium are incubated in darkness at 25°C, whereas suspension cultures are incubated in semi-darkness.

Maturation. Although Pliego-Alfaro and Murashige (1988) and Mooney and van Staden (1987) reported that somatic embryo development followed the transfer of embryogenic cultures on to semi-solid medium without picloram, the absence of

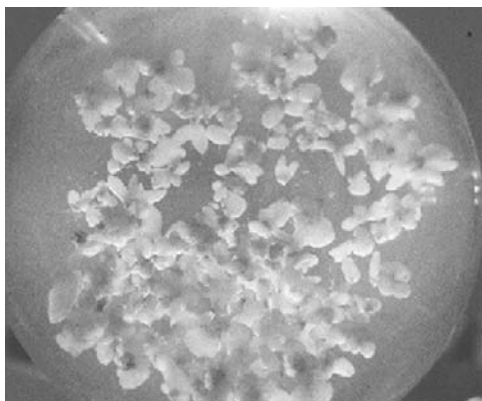


Fig. 11.1.1.1. Cotyledonary stage avocado somatic embryos in liquid medium.

auxin is not essential for somatic embryo development of SE-type cultures (Witjaksono and Litz, 1999a; Fig. 11.1.1). This has been confirmed by Raviv *et al.* (1998), who observed that cotyledonary somatic embryos develop on semi-solid medium supplemented with $9.04 \mu\text{M}$ 2,4-dichlorophenoxyacetic acid (2,4-D) and $2.22 \mu\text{M}$ BA. Somatic embryo development following transfer from maintenance to maturation media is genotype-dependent, and the development from SE-type cultures is more efficient (Witjaksono and Litz, 1999a,b).

Development of somatic embryos is also mediated by a few physical parameters (Witjaksono and Litz, 1999b), e.g. gelling agent and sucrose concentration, which affect both the size and number of good-quality somatic embryos. Although the largest number of somatic embryos develops on medium supplemented with 6–7 g/l gellan gum and 90 g/l sucrose, their size is reduced at this concentration of sucrose. Hyperhydricity is invariably a consequence of optimizing maintenance in suspension culture, and somatic embryo development on medium with high gellan gum and sucrose concentrations can reverse or prevent this physiological disorder. The optimum growth conditions for somatic embryo maturation include MS medium which has been supplemented with 30 g/l sucrose, 4 mg/l thiamine HCl, 100 mg/l myoinositol and 6.0 g/l gellan gum in darkness at 25°C . Addition of 20% (v/v) filter-

sterilized coconut water increases the frequency of recovery of high-quality somatic embryos (Witjaksono and Litz, 2002).

Germination. Fully enlarged, opaque mature somatic embryos (0.8–1.0 cm) are transferred to semi-solid germination medium, which is MS medium supplemented with $4.44 \mu\text{M}$ BA and $2.89 \mu\text{M}$ GA_3 (Witjaksono and Litz, 1999b). After 6 months, somatic embryos develop shoots only or roots only or are bipolar. Shoot development is generally low due to failure of the apical meristem to become organized (Pliego-Alfaro and Murashige, 1988). Maturation medium supplemented with filter-sterilized coconut water can increase the frequency of recovery of somatic embryos with apical meristems (Witjaksono and Litz, 2002; F. Pliego-Alfaro, personal communication).

Witjaksono *et al.* (1998) rescued somatic embryo shoots by micropropagating them according to the protocol described above (Witjaksono *et al.*, 1999b). Another procedure that has been successfully exploited has involved the micrografting of somatic embryo-derived shoots on decapitated seedlings (Raharjo and Litz, 2003).

Individual micropropagated shoots derived from somatic embryos can be rooted according to the induction and development procedure of Pliego-Alfaro (1988) and Witjaksono *et al.* (1999b). Individual shoots (1.5–2 cm length with one to three leaf primordia and non-expanded leaves) that develop from proliferating shoot cultures are cultured for 3 days on MS medium supplemented with $122.6 \mu\text{M}$ IBA. Culture conditions include a 16 h photoperiod ($100\text{--}120 \mu\text{mol}/\text{m}^2/\text{s}$ provided by cool white fluorescent bulbs) at 25°C .

5.1.2. Protoplast isolation and culture

The first reports of isolation and culture of avocado protoplasts involved studies of ASBVd using callus-derived protoplasts (Blickel *et al.*, 1986) and fruit ripening studies using protoplasts from fruit mesocarp (Percival *et al.*, 1991). These studies utilized non-morphogenic cultures. Witjaksono and Litz (2000) recovered protoplasts from non-

morphogenic callus suspensions of two *Persea* spp. in the subgenus *Eriodaphne*, i.e. *P. cinerascens* and *P. pachypoda*. Witjaksono *et al.* (1998) described the isolation and culture of protoplasts from embryogenic avocado cultures, and regenerated plants from somatic embryos. Avocado protoplasts have been successfully cultured in liquid (Witjaksono *et al.*, 1998) and in agarose-solidified (Witjaksono *et al.*, 1999a) media, although the former procedure is more efficient. Yields of $> 3 \times 10^6$ protoplasts/g are obtainable from embryogenic cultures maintained in liquid medium. The isolation and culture of protoplasts and their regeneration are genotype-dependent, and the procedure is more efficient with PEM-type cultures than with SE-type cultures. Development of PEMs from protoplasts in liquid medium is dependent on medium osmolarity, nitrogen source and plating density. PEMs develop in medium with 0.4 M osmolarity, while microcalluses develop in medium with 0.6 M osmolarity. The optimum medium and conditions for recovery of PEMs from protoplasts consist of 0.4 M MS-8P with a 0.8×10^5 /ml protoplast density.

Protoplasts are cultured in 2–3 ml liquid medium in sealed 60 mm $\times$ 15 mm sterile plastic dishes, and maintained in darkness at 25°C. Approx. 5% of protoplasts divide after 5 days of culture, and the plating efficiency after 12 days is approx. 25%. Protoplast-derived PEMs are able to develop on maturation medium (see Section 5.1.1. above), and mature somatic embryos have germinated, albeit with a low conversion frequency.

5.2. Genetic manipulation

5.2.1. Mutation induction and somaclonal variation

Breeding objectives. Somatic mutations of avocado that affect tree architecture, leaf size, leaf shape and colour, fruit size and shape, and skin texture have been recognized. Several off-types of 'Fuerte', including 'Weisel', 'Newman' and 'de Bard', have been recovered (Hodgson, 1945). The rootstock selection 'D9' originated from irradiated 'Duke', and has good resistance to PRR,

together with some dwarfing effect on the scion. Scions grafted on 'D9' are more productive than those on 'Martin Grande' but less so than those on 'Borchard' and 'Duke 7' (Arpaia *et al.*, 1992). 'Hass' that was irradiated with ^{60}Co showed reduced vegetative growth, and greater flowering and fruit set were observed from 4-year-old plants exposed to 13 Gy (de la Cruz-Torres *et al.*, 1995a). Following irradiation at 15 Gy, there was variability in height, rootstock and graft diameter, stomatal density and internode length (de la Cruz-Torres *et al.*, 1995b).

Witjaksono and Litz (2004) reported the radiation sensitivity of embryogenic cultures of 'Fuerte' and 'T362'. The effects of irradiation on embryogenic suspensions and on somatic embryo development from irradiated cultures were described. The approximate PD_{50} as determined by linear regression was 35 Gy 2 weeks after irradiation for 'Fuerte' and 4 weeks after irradiation for 'T362'. Irradiation did not significantly affect the number of early stage 'Fuerte' 2.11.1 somatic embryos that developed from irradiated cultures; however, 10–50 Gy inhibited somatic embryo development. Irradiation of 'T362' embryogenic cultures at 25–50 Gy inhibited the number of intermediate and mature stages of somatic embryos that developed from irradiated cultures, and 50 Gy inhibited somatic embryo maturation. Irradiation up to 10 Gy significantly increased the number of mature 'Fuerte' somatic embryos that developed from suspension cultures. Irradiation with doses up to 25 Gy stimulated development of heart stage 'T362' somatic embryos; however, mature somatic embryo development was suppressed at dosages of 10 Gy and greater. The aim of this study is to select *in vitro* for resistance to the culture filtrate of *P. cinnamomi* and to regenerate plants that may have resistance to PRR (Witjaksono, 2001).

5.2.2. Somatic hybridization

Breeding objectives. The immunity to PRR that is associated with species in subgenus *Eriodaphne* is inaccessible to plant breeders

due to graft and sexual incompatibility barriers between *Persea* spp. in this group and with species in subgenus *Persea* (Frohlich *et al.*, 1958). Pliego-Alfaro and Bergh (1992) suggested that somatic hybridization might be the appropriate way to achieve hybridization between species in the two subgenera.

Protocol. Witjaksono (1997) reported the attempted somatic hybridization of avocado by means of protoplast fusion involving protoplasts from embryogenic avocado cultures with leaf protoplasts of PRR-resistant species. In order to assure a constant supply of protoplasts, a procedure for micropropagating *Persea* species (subgenus *Eriodaphne*) from *in vitro*-germinated seedlings was developed (Witjaksono, 1997). The results of this study were inconclusive; however, somatic hybrids were recovered as a result of the fusion of embryogenic avocado protoplasts with non-morphogenic protoplasts of *Persea* spp. in subgenus *Eriodaphne* (Witjaksono and Litz, 2000); however, plants were not regenerated from the hybrid somatic embryos.

5.2.3. Genetic transformation

Breeding objectives. Genetic transformation could be used to address some important rootstock and scion breeding objectives. A primary breeding objective has been to develop improved avocado rootstock cultivars with greater resistance to PRR and scion cultivars with enhanced resistance to foliar and fruit diseases, using genes that encode for disease resistance or pathogenesis-related proteins. The control of fruit ripening is another important breeding objective that would have two important ramifications: (i) enable the on-tree storage of West Indian and West Indian $\times$ Guatemalan avocados; and (ii) extend the postharvest storage time of all types of avocados.

Protocol. The genetic transformation of avocado has been based upon highly embryogenic suspension cultures, described above. Growth of PEM-type embryogenic

suspensions can be suppressed by 50% with 50 mg/l kanamycin sulphate, whereas 50% growth suppression on semi-solid medium requires 100 mg/l kanamycin sulphate (Cruz-Hernandez *et al.*, 1998). Complete suppression of growth of embryogenic cultures occurs on semi-solid medium containing 200 mg/l kanamycin sulphate.

Cruz-Hernandez *et al.* (1998), utilizing PEM-type cultures, described a two-step selection procedure for recovery of genetic transformants. PEM-type embryogenic cultures growing on semi-solid maintenance medium were gently abraded with a soft camel-hair brush. The abraded embryogenic cultures were then incubated with acetosyringone-activated *Agrobacterium tumefaciens* (strain 9749 ASE2 containing a co-integrate vector pMON9749 with a selectable kanamycin-resistant marker (*nptII*) and β -glucuronidase (GUS)) in liquid maintenance medium, and co-cultured for 3 days at 100 rpm. *A. tumefaciens* was then eliminated by incubating the cultures in maintenance medium supplemented with 50 mg/l kanamycin sulphate and 200 mg/l cefotaxime. After an initial selection for antibiotic resistance in liquid maintenance medium containing 50 mg/l kanamycin sulphate for 2–4 months, there was a second, more intensive selection in the presence of 100 mg/l kanamycin sulphate for 2 months. Finally, the cultures were cultured in 200 mg/l kanamycin sulphate to eliminate chimeras. Somatic embryo development was initiated by subculture of transformed PEMs on to maturation medium without kanamycin sulphate, followed by subculture on to maturation medium containing kanamycin sulphate. Transformed somatic embryos stained positively for GUS (Jefferson, 1987), and the integration of *nptII* and GUS into the avocado genome was confirmed by PCR and Southern hybridization (Miller, 1972; Doyle and Doyle, 1990). Transgenic plants were not regenerated.

Accomplishments. Using a modification of the protocol described above, it has been possible to genetically transform avocado with genes that could affect different horti-

cultural traits. The strategy that has been adopted for controlling avocado fruit ripening is based upon blocking ethylene production with the *S*-adenosyl-L-methionine (SAM) hydrolase (SAMase) gene, which converts SAM to a non-toxic by-product that it is not available to be converted into ACC (Good *et al.*, 1994). In this manner, the biosynthesis of ethylene is blocked. The SAMase gene is in pAG4092 under the control of an avocado fruit-specific cellulase promoter with *npHII* as a selectable marker. Efendi (2003) has described the recovery of transgenic plants that contain the SAMase gene.

Embryogenic avocado cultures have also been genetically transformed with pathogenesis-related (PR) genes, including β -1,6-glucanase, chitinase and the antifungal protein (AFP) gene, in order to address the problem of PRR of avocado rootstocks (S. Raharjo and Witjaksono, Homestead, USA, personal communication; Figs 11.1.2–11.1.4). The AFP gene is in pGPTV-BAFP together with *uidA*, the bar gene for resistance to phosphinothricin and the cauliflower mosaic virus (CaMV) 35S promoter. Glucanase and chitinase have been cloned in pGPTV-BCG, together with *uidA*, *bar* and the CaMV 35S promoter. The initial greenhouse and field trials commenced in 2003 (Raharjo *et al.*, 2003).

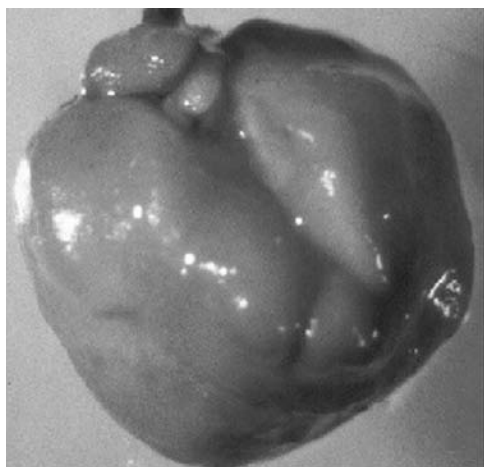


Fig. 11.1.2. Genetically transformed mature avocado somatic embryo, demonstrating the presence of GUS.



Fig. 11.1.3. Transgenic avocado plant *in vitro*-regenerated from somatic embryo.



Fig. 11.1.4. Genetically transformed avocado plants *ex vitro*.

5.3. Cryopreservation

Long-term conservation of avocado genetic resources is confounded by the high costs of land, labour and grove management, including the control of pests and diseases, and by inclement weather. Genetic diversity within the genus *Persea*, within the subgenera *Persea* and *Eriodaphne* and within *Persea americana* is large, so that meaningful collections of species, subspecies and cultivars for breeding and genetic studies must be extensive. Manipulation of avocado at the somatic cell level requires a constant supply of embryogenic material. There is a strong genotype-

dependent loss of embryogenic competence *in vitro* (Witjaksono *et al.*, 1999a). Therefore, the annual renewal of embryogenic lines has been essential, resulting in the expenditure of considerable time and expense. Cryopreservation could resolve some of the problems associated with ensuring a continuous supply of embryogenic cultures. In addition, cryopreservation could impact the long-term maintenance of clonal *Persea* genetic resources by providing a backup for existing collections.

Efendi *et al.* (2001), Efendi and Litz (2002, 2003) and Efendi (2003) have described the recovery of embryogenic avocado cultures from cryogenic storage, and successfully regenerated somatic embryos from these cryopreserved cultures. Two procedures were described: (i) stepwise cooling ($-1^{\circ}\text{C}/\text{min}$ from room temperature to -75°C followed by rapid cooling to -196°C ; and (ii) rapid cooling (vitrification) from room temperature to -196°C . For stepwise cooling, embryogenic cultures were treated with different cryoprotectant combinations consisting of dimethyl sulphoxide (DMSO) and glycerol (5% + 5%, 10% + 10% and 15% + 15% (v/v)), and cooled at the rate of $-1^{\circ}\text{C}/\text{min}$ to -75°C in 'Mr Frosty' containers. The cryovials were removed and were plunged into Dewar flasks containing liquid nitrogen. For cryopreservation by vitrification, embryogenic cultures were suspended in plant vitrification solution 2 (PVS2) (Sakai *et al.*, 1991), consisting of glycerol (30%), ethylene glycol (15%) and DMSO (15%), for 15 min prior to rapid cooling by plunging into liquid nitrogen. Following the removal of vials from liquid nitrogen and rapid warming to room temperature, cultures were thoroughly washed with maintenance medium and plated on semi-solid maintenance medium formulation. Somatic embryo development was initiated by subculturing on somatic embryo maturation medium.

4. Conclusions

A few groups in major avocado-producing countries (USA, Spain, Israel, Mexico and Australia) have made an early and signifi-

cant impact on avocado improvement using modern genetics and cell culture techniques. Perhaps because avocado has been a good model for study of the basic physiology of fruit ripening, there have been a number of published and unconfirmed reports of gene cloning of the important genes implicated in this process. Moreover, two of the major breeding goals for avocado are fairly well defined: control of fruit ripening and control of PRR. These objectives are attainable using current genetic transformation procedures. It is probable that the avocado PRR problem can also be addressed using *in vitro* mutation induction and selection. There appears to be a strong readiness among groups to collaborate, which should enable steady and rapid progress.

Biotechnology can address other important issues associated with this crop. The use of either shoot tip and nodal culture or somatic embryogenesis as propagation procedures could resolve some of the serious limitations of existing methods for vegetatively propagating PRR-resistant rootstocks by the etiolation method (Frolich and Platt, 1972). Control of avocado sunblotch disease caused by a viroid could possibly be addressed by genetic transformation with the *pac1* ribonuclease gene, which has been demonstrated to be highly effective against potato spindle tuber viroid (Sano *et al.*, 1997).

It is expected that important genes will be identified, studied and eventually applied to the avocado. Major discoveries will occur in the area of genomics research, which is a new platform of technologies aiming at understanding whole genomes, e.g. large-scale sequencing, bioinformatics, DNA chips, etc. The function of various genes as well as the interactions between them will be disclosed. Specifically for avocado, it is our expectation that more avocado genes will be identified directly by these tools and by establishing homologies with genes from other plants in which application of these technologies is more advanced (*Arabidopsis*, maize, rice and tomato). Identification of the genes coding for important traits, including yield, fruit size and shape, will, on the one hand, improve classical breeding by marker-assisted selection and, on the other, provide

suitable genes for generation of transgenic plants with the desired characters.

Cryopreservation of embryogenic avocado lines can have great utility not only to ensure a supply of morphogenetically competent cells, but also for use as a backup for avocado germplasm collections and to facilitate the international movement of important genetic resources, particularly from the great genetic repositories in the USA, Israel, Mexico and Australia.

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12

Moraceae

The family *Moraceae* is within the division *Magnoliophyta*, class *Magnoliopsida*, subclass *Hamamelidae* and order *Urticales*. It consists of 40 genera and 1400 species, which are monoecious or dioecious trees, shrubs, lianas and, rarely, herbs (Watson and Dallwitz, 1992 onwards). *Moraceae* members are distributed

mainly in the tropics and subtropics with a few species in the temperate regions. Only the genera *Morus*, *Ficus* and *Artocarpus* contain economically important sources of food and *Morus* spp. are also important in sericulture (Anon., 1956).

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12.1 *Ficus carica* Fig, *Artocarpus* spp. Jackfruit and Breadfruit and *Morus* spp. Mulberry

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1. Introduction

1.1. Botany and history

1.1.1. *Morus*

The genus *Morus* includes 68 species and is believed to have originated in the Himalayan belt in the Indo-China region (Sanjappa, 1989). *M. alba* L. and *M. indica* L. (mulberry) produce edible fruit and are also the most important species for sericulture. Mulberry is a rapidly growing deciduous woody perennial having a deep root system. The plant has simple, alternate, stipulate, petiolate, entire or lobed leaves. The inflorescence is a catkin, containing a pendent or drooping peduncle bearing unisexual flowers. Male catkins are usually longer than the female catkins. It has four perianth lobes and four stamens implexed in the bud. The female inflorescence is usually short and the flowers are very compactly arranged. The ovary is one-celled and the stigma is bifid. The fruit is a sorosis, mainly violet black in colour (Datta, 2000). Leaves of *M. alba* have antioxidant activity due to the presence of β -carotene and α -tocopherol (Yen *et al.*, 1996). Hypoglycaemic activity in *M. indica* leaves has been determined (Kelkar *et al.*, 1996). Paper mulberry (*Broussonetia papyrifera* L. Ventenat), which resembles mulberry mor-

phologically, is cultivated in Japan for its bark and is used as a raw material for making paper. The best silk is obtained from the silkworm *Bombax mori*, which feeds only on mulberry leaves. The silkworm is attracted to the mulberry leaf fragrance and is equipped with special organs, which respond to the taste of the leaves (Oka and Ohyama, 1986). Silk proteins, which are composed of fibroin and sericin, are obtained from mulberry leaves and the leaf sugars (30 and 20% dry weight (DW) of the leaves) impart the taste, making it a good diet for silkworms. The preferential food value of the mulberry leaf for silkworm larvae is attributed to the presence of three stimulant factors, i.e. an attractant, a biting factor and a swallowing factor. These substances (approx 0.2% in leaves) have been identified as citral, linalyl acetate, linalol and terpinyl acetate. Hexenol β -sitosterol, along with some sterols and a water-soluble substance, is the main factor which stimulates biting (Singh and Makkar, 2001).

1.1.2. *Ficus*

The genus *Ficus* is remarkable for the variation among its 13 species, and is widely distributed throughout the tropics and subtropics of both hemispheres. Several species produce edible figs of varying palatability,

whereas many other arborescent *Ficus* species are cultivated for shade, as avenue trees and as ornamentals. *F. carica* L. is considered to be native to south-western Asia and to have been brought into cultivation in the southern Arabian peninsula by 3000 BC (Anon., 1956). The fig tree is small or moderate in size, and deciduous with broad, ovate, three- to five-lobed leaves. The leaves are rough above and pubescent below, long stalked and dark green with pronounced venation. The female fig plants have larger leaves and more spreading crowns than male trees (Sadhu, 1992). *F. carica* is a gynodioecious species having two distinct forms of trees. Flowers are minute and, together with numerous thin branches, cover the inner surface of a hollow globose or pear-shaped receptacle. The fruit is a syconium, a fleshy hollow receptacle with a narrow aperture at the tip and several small male, female and gall-type flowers lining the inner surface. True fruits are the tiny droplets inside the cavity of the fused peduncle. Depending upon the nature of the flowers and the pollination method, there are four classes of figs: (i) common or Adriatic fig with pistillate flowers and fruits that develop without stimulation of pollination; (ii) Capri fig, which is most primitive and inedible; (iii) Smyra fig, whose fruits do not develop unless flowers are pollinated; and (iv) San Pedro fig, which is an intermediate type, i.e. the first crop is parthenocarpic and the second is only after pollination (Anon., 1956).

The Smyra fig is the most important type and is extensively grown in the Mediterranean region. The figs are consumed fresh, dried, preserved, candied or canned. The fig is fairly salt- and drought-tolerant (Sadhu, 1992). Soils of high lime content produce fruits of better quality suitable for drying. Fig trees can also withstand temperatures as low as -12 to -9.5°C . World production of fig in 2001 has been estimated to be $>988,000$ t (FAOSTAT, 2004). The leading producers include Turkey (280,000 t), Egypt (188,039 t), Greece (80,000 t), Iran (77,000 t) and Morocco (67,000 t). Figs are readily cultivated by hardwood cuttings although budding, grafting and air layering are also successful. The fig tree bears twice a

year. Pruning is used to induce growth of flower-bearing wood and thereby improve the fruit yield. Pruning also increases the fruit weight in early cultivars.

1.1.3. *Artocarpus* spp.

The genus *Artocarpus* consists of approx. 50 species of small to large evergreen trees and is native to South and South-east Asia. *A. altitis* (Parkinson) Fosberg (breadfruit) and *A. heterophyllus* Lamk. (jackfruit) are grown mainly for their edible fruits, and are cultivated throughout the tropics and in many parts of the subtropics (Rajendran, 1992; Soepadmo, 1992). Production data are not available for any of the *Artocarpus* fruits. The trees are of medium size, 8–10 m tall, having a dense irregular globose crown. The leaves are dark green, alternate, petioled, ovate-oblong or obovate. Other species, including *A. chaplasha*, *A. hirsutus* and *A. lakoocha*, are important timber trees. The inflorescence is solitary and is borne in the axil of the leaf with barrel-shaped male flower heads. Flowers are a mixture of sterile and fertile ones, closely embedded on a receptacle. Fertile male flowers are tubular and bilobed, with a 1–1.5 mm long perianth and 1–2 mm long stamens. Female heads are borne singly or in pairs, distal to the position of male heads. They are cylindrical or oblong, dark green, 5–15 cm long and 3–4 cm across, with a distinct annulus at the top end of the stout stalk. Female flowers have a tubular perianth, fused at both ends, and project as angular (3–7 angles), blunt or pointed and topped with spatulate or ligulate styles and stigmas. All species possess syncarpic fruit, i.e. edible, aggregate fruit. The axis of the inflorescence, the ovaries and the perianths all grow simultaneously and develop into a multiple fruit called a sorosis.

Distinct cultivars of jackfruit are not known. Cultivated jackfruit is broadly classed into two groups, i.e. (i) soft flesh, with juicy, soft and sweet to sweet-acid pulp; and (ii) firm flesh, with sweet, firm and crispy pulp. The pericarp around each seed and the fleshy perianth are edible. Mature trees range from 9 to 25 m in height with a straight stem branching near the base to

form a dense, irregular crown (Jaiswal and Amin, 1992). The large fruit (40–50 kg) is rich in vitamin A, carbohydrates and proteins. Jacalin, a lectin derived from jackfruit seeds, exhibits immunobiological activity (Kabir, 1998).

There are two distinct breadfruit varieties: seeded and seedless. The seeded breadfruit variety is considered to be the wild form. The seedless fruit depends on pollination to stimulate parthenocarpic growth. Although so-called seedless forms occasionally produce fruits with some seeds, seedlessness is mainly caused by mismatching of time of pollen anthesis and formation of female flowers ready for fertilization (Rajendran, 1992). The seedless variety is popularly cultivated and contains a mass of whitish edible pulp, which is cooked and consumed as a starchy staple.

1.2. Breeding and genetics

1.2.1. *Morus*

Mulberry ($2n = 2x = 28$) breeding has mainly been directed towards identification of high-yielding and nutritious cultivars suitable for different soil and climatic conditions and agronomical practices, with uniform growth and resistance to pests and diseases. The main thrust of breeding is on clonal selection, hybridization, introduction of polyploids, mutation breeding, recurrent selection and polycrossing to screen high-yielding and nutritive lines (Shoukang *et al.*, 1987; Jain *et al.*, 1996; Ma *et al.*, 1996; Islam and Joarder, 1997; Chakraborti *et al.*, 1999). Scions are selected from high-yielding, rapidly growing types with large leaves of good food value.

1.2.2. *Ficus*

Although figs are extensively cultivated, very little work has been done for its improvement. The common fig is diploid ($2n = 2x = 26$). Most existing cultivars are selections made from wild seedling trees and chance seedlings. According to Sadhu (1992) the objectives of present-day fig breeding

include: (i) development of high-yielding cultivars; (ii) improvement in fruit quality; (iii) elimination of caprification; and (iv) transfer of nematode and insect resistance characters of wild fig to high-yielding, good-quality cultivars. These cultivars have since been maintained through cuttings. Attempts at interspecific hybridization have yielded few hybrids of commercial value. In California, USA, 11 new hybrids were obtained with desirable fruit characters and have been adopted for commercial cultivation (Sadhu, 1992). In Louisiana, USA, root knot nematode-resistant 'Hunt' and 'Celeste' have been obtained through breeding and controlled pollination of bagged spring and summer Capri fig syconia, resulting in sweeter figs (Sadhu, 1992). Within the Sardinian fig germplasm, > 30 cultivars have been described and characterized according to their morphological characters. Most of them are autochthonous and all are of the 'common type' (Anon., 1956).

1.2.3. *Artocarpus*

Despite the importance of jackfruit in South and South-east Asia and of breadfruit in South-east Asia and Polynesia, there has been no breeding with either of these crop species despite the apparent genetic heterogeneity within the genus (Rajendran, 1992; Soepadmo, 1992). Breeding objectives would include trees bearing a large number of small- to medium-sized fruits and which have more pulp, smaller and fewer seeds and thinner skin. The chromosome number of jackfruit is $2n = 4x = 56$ and that of breadfruit is $2n = 2x = 27$ or 28 and $2n = 4x = 56$.

2. Molecular Genetics

2.1. Gene cloning

In the *Morus* spp., steroids and isoprenoid compounds in leaves mainly contribute to silkworm health and silk production. The production of isoprenoids from mevalonate is catalysed by the enzyme hydroxymethylglutaryl coenzyme A (CoA) reductase (HMGR). The HMGR genomic clone, *Mahmg1*, has been

isolated from *M. alba* and its expression has been characterized (Jain *et al.*, 2000). Alteration in this pathway would in turn alter the production of silk by silkworm larvae.

2.2. Molecular markers

Cultivar identification of *Morus* spp. has been achieved using peroxidase (PRX) isozyme markers (Hirano, 1977). Assessment of fig genotypes using isozymes has been attempted (Khadari *et al.*, 1995; Elisiario *et al.*, 1998). Fig cultivars have been characterized with isozymes. Seventeen enzyme systems have been analysed and acid phosphatase (AcPH), diaphorase (DIA), fumarase (FUM), glutamate oxalacetate transaminase (GOT), malate dehydrogenase (MDH), PRX and phosphoglucosomerase (PGI) have shown high resolution of the bands and reproducibility. All these enzymes except FUM (which had a monomorphic pattern) proved to be useful to allow characterization of almost all fig varieties (Chessa *et al.*, 1998). Similar studies using random amplified polymorphic DNA (RAPD) markers to assess fig genotypes have been reported by Khadari *et al.* (1995) and Elisiario *et al.* (1998).

Schnell *et al.* (2001) reported that amplified fragment length polymorphism (AFLP) markers could be utilized to measure the genetic diversity in a jackfruit germplasm collection. They observed that 12 primer pairs could be used to screen the collection, and 187 AFLP markers were obtained. Using cluster analysis and principal component analysis, the study demonstrated that accessions from India and from South-east Asia could be distinguished from each other.

Phylogenetic relationships between 11 *Artocarpus* species, jackfruit and breadfruit have been studied by restriction fragment length polymorphism (RFLP) analysis of an amplified region of chloroplast DNA (cpDNA) (Kanzaki *et al.*, 1997). Thirty restriction site mutations were compared, and data were analysed by neighbour-joining and parsimony analysis. The results indicated that jackfruit and *A. integer* Merr. are monomorphic and indistinguishable for all restriction sites, and therefore closely related. Breadfruit

and *A. elasticus* formed a monophyletic group, and supported the hypothesis that the breadfruit originated from the latter species.

3. Micropropagation

3.1. Morus

Micropropagation has been successful with a few mulberry cultivars (Susheelamma *et al.*, 1996). Oka and Ohyama (1981) first reported regeneration of plants from axillary buds of *M. alba*. Subsequently, Patel *et al.* (1983) regenerated *M. indica* from axillary buds from mature trees. Suzuki and Kohno (1987) reported that pinching and defoliation stimulated sprouting of lateral buds. Mhatre *et al.* (1985) pre-treated *M. indica* axillary buds in benzyladenine (BA)-supplemented liquid medium to stimulate multiple shoot formation (Fig. 12.1.1a). Multiple shoots have been regenerated *in vitro* from axillary buds of *M. alba* (Katase *et al.*, 1986; Kim *et al.*, 1986; Oka and Ohyama, 1986; Narayan *et al.*, 1989; Chitra and Padmaja, 1999), *M. australis* (Pattnaik *et al.*, 1996), *M. bombycis* (Oka and Ohyama, 1986; Jain and Datta, 1992), *M. indica* (Mhatre *et al.*, 1985), *M. kagayamae* (Oka and Ohyama, 1986), *M. latifolia* (Oka and Ohyama, 1986) and of several mulberry genotypes (Jain *et al.*, 1990).

M. alba 'Mandalaya' is commercially propagated from individual axillary buds on semi-solid Murashige and Skoog (1962) medium (MS) supplemented with 5.71 μ M indoleacetic acid (IAA) and 8.87 μ M BA; formation of multiple buds occurs and plants are regenerated following transfer to MS with 5.37–10.74 μ M naphthaleneacetic acid (NAA) for rooting (Chattopadhyaya *et al.*, 1990). Use of agitated liquid medium for micropropagation of mulberry has also been reported (Tewary and Oka, 1999).

Mulberry regenerants grown under field conditions have been evaluated (Chakraborti *et al.*, 1999). Greater leaf biomass has been observed in plants derived from *in vitro* culture compared to plants obtained through cuttings (Patel *et al.*, 1983). Islam and Joarder (1997) conducted a comparative study using

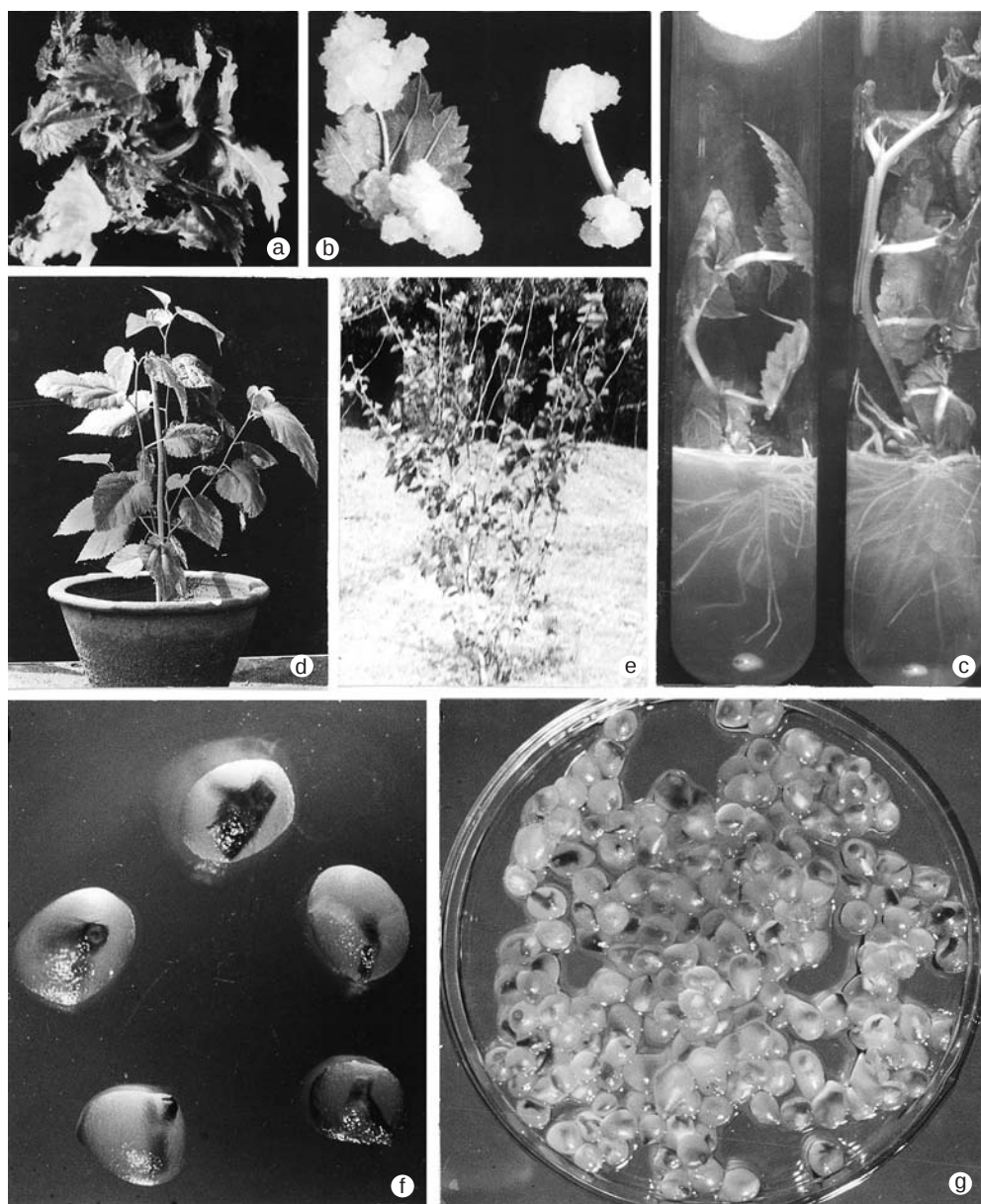


Fig. 12.1.1. Plant regeneration *in vitro* and synthetic seeds of mulberry (*Morus indica* L.): (a) multiple shoots from axillary buds; (b) initiation of callus from leaf and stem of mature plant; (c) rooted plantlet ready for acclimatization; (d and e) acclimatized plants; (f) encapsulated buds (close view); (g) encapsulated buds (general view).

morphological and biochemical tests among established micropropagated and cutting-derived plants of mulberry and concluded that micropropagated plants had greater vigour.

3.2. *Ficus*

Shoot tip/nodal culture has been reported for several *Ficus* spp., e.g. *F. auriculata* (Muriithi *et al.*, 1982; Amatya and Rajbhandary, 1989),

F. religiosa (Nagaraju *et al.*, 1998), *F. benjamina* (Kristiansen, 1992) and *F. lyrata* (Jona and Gribaudo, 1987). Shoot tips (Pontikis and Melas, 1986, as cited in Karale *et al.*, 2001) and meristem explants (Barbosa *et al.*, 1992, as cited in Karale *et al.*, 2001) have been used for *in vitro* establishment of fig. Shoots have been produced from shoot tips of 'Kalamon' on semi-solid MS with 0.5 μM phloroglucinol, and were rooted on MS with 4.9 μM indolebutyric acid (IBA) (Pontikis and Melas, 1986, as cited in Karale *et al.*, 2001). Barbosa *et al.* (1992, as cited in Karale *et al.*, 2001) obtained plantlet regeneration from shoot tips on MS medium containing activated charcoal. They also reported success with 'Berbera' and 'Lampa' on medium containing polyvinylpyrrolidone (PVP) (0.025, 0.05 or 1%) or 56.8 or 113.6 μM ascorbic acid. Nodal explants have been cultured on MS with 0.7, 1.1 or 2.2 μM BA alone or in combination with 1.0 μM NAA. Optimum shoot multiplication occurs on 2.2 μM BA alone (Nobre and Ramano, 1998, as cited in Karale *et al.*, 2001). Demiraley *et al.* (1998, as cited in Karale *et al.*, 2001) worked with 'Bursa Siyahi', and obtained shoot development on MS supplemented with 2.22 μM BA, 0.49 μM IBA and 0.29 μM gibberellic acid (GA_3) with or without 89 mg/l phloroglucinol or 2 g/l activated charcoal. Kumar *et al.* (1998) reported the use of apical buds from a 7–8-year-old tree, and produced multiple shoots on MS with 8.87 μM BA and 1.07 μM NAA.

3.3. Artocarpus

Shoot tip/nodal culture of jackfruit using seedling and mature tissues has been reported (Rao *et al.*, 1981; Rahman, 1988a,b; Rahman and Blake, 1988a,b; Roy and Hadiuzzaman, 1991; Roy *et al.*, 1992; Amin and Jaiswal, 1993; Rouse-Miller and Duncan, 2000). Amin and Jaiswal (1993) developed a rapid vegetative propagation procedure using apical buds excised from new shoots from the trunk of mature jackfruit trees. Buds obtained from emerging trunk sprouts produced clumps of multiple shoots, whereas buds obtained from actively growing top branches generally elongated to form

solitary shoots. Use of MS supplemented with 4.5–9 μM BA and kinetin (separately or together) supported shoot proliferation. Amin and Jaiswal (1993) observed that shoots in the initial subcultures were difficult to root, but shoots that developed later could be rooted with 60–80% success on half-strength MS with 10 μM IBA. *In vitro* shoots derived from jackfruit seedlings can be rooted without auxins (Rahman and Blake, 1988b).

Regeneration of breadfruit from shoot tips of mature trees has been recently documented (Rouse-Miller and Duncan, 2000) using 2.2 μM zeatin.

4. Virus Elimination

Haelterman and Docampo (1994, as cited in Karale *et al.*, 2001) standardized a technique for *in vitro* propagation of mosaic-indexed fig using thermotherapy and shoot tip culture. Stem cuttings (1 cm diam.) with mosaic symptoms were rooted and subjected to thermotherapy (37°C for 20 days). Buds obtained from these cuttings were grown on semi-solid MS with 0.88 μM BA, 2.32 μM kinetin and 0.29 μM GA_3 . The proliferation rate was 1.4–1.6 every 45 days. Shoots developed with vigorous root primordia in 15–20 days on half-strength MS with 9.8 μM IBA.

5. Somatic Cell Genetics

5.1. Regeneration

5.1.1. Organogenesis

Morus. Saito and Katagiri (1989) developed a single-step adventitious bud induction method from mulberry leaves excised from winter or lateral buds. Subsequently, adventitious bud formation on cultured immature leaves and on leaves of *in vitro* mulberry shoots has been reported (Oka and Ohyama, 1981; Machii, 1992a; Saito and Katageri, 1992; Yamanouchi *et al.*, 1993, 1999). Optimum culture conditions have been reported by Minamizawa and Hirano (1974b) and Yamanouchi *et al.* (1999) for adventitious bud

formation using immature leaves of winter buds. Sawaguchi *et al.*, (1997) examined the effects of culture conditions on adventitious bud formation from cotyledons and primary leaves excised from mulberry seedlings. The basal leaf portion produced the maximum number of shoot buds on medium containing BA (Sugimura *et al.*, 1998). Machii (1999) studied selected mulberry genotypes having a high morphogenic potential by screening immature leaves of 287 genotypes on MS medium containing BA; IAA was used for rooting. Adventitious buds formed in 121 genotypes and developed shoots in 83 genotypes, and plants were regenerated in 55 genotypes. A cyclic production of buds was possible by repeatedly refreshing the cultures from which the regenerated shoots were removed and the original immature leaf explants were recultured. Mhatre *et al.* (1985) reported that pre-treatment (soaking) of leaves in BA-supplemented liquid medium for specific time intervals stimulated adventitious shoot formation with *M. indica*. The maximum number of shoot buds was produced on leaf explants cultured in an upright position in the culture medium. Type of sugar can affect differentiation from stem segments (Minamizawa and Hirano, 1974a). Leaves of *M. nigra* without petioles from cultured shoots, soaked in liquid MS medium with BA for 48 h prior to culture on solidified hormone-free medium, produce multiple shoots.

Maintenance. Regeneration of plants has been reported by Pratap *et al.* (1989), Yusa and Watanabe (1989), Sahoo *et al.* (1997), Roy (1999) and Vijayan *et al.* (1999) from callus derived from leaf and stem segments. Machii (1992a) observed that the composition of the basal medium influences the nature of the callus and that the regeneration of plants in callus cultures is genotype-dependent. This was also observed in *Morus indica* (Fig. 12.1.1b). A strong genotypic and hormonal influence on all developmental phases of callus growth has been demonstrated by Susheelamma *et al.* (1996) and Bhau and Wakhlu (2001).

Suspension cultures can be initiated from various explants of mulberry (Oka and Ohyama, 1973). Yamada and Okamoto (1977) initiated large-scale suspensions of cells

derived from leaf veins and reported a growth index of 3–4 over 2 weeks on IBA-supplemented medium. Presoaked internodal explants in BA produced loose and nodular organogenic cultures (Jain and Datta, 1992).

Rooting. Shoots derived from adventitious buds can be rooted by subculture on to semi-solid MS medium with 0.98 to 4.90 μM IBA and activated charcoal (0.3%); plantlets have been established in soil (Yadav *et al.*, 1990). Rooted plantlets of *Morus indica* have been successfully acclimatized (Fig. 12.1.1c–e).

Artocarpus. Rao *et al.* (1981) reported shoot regeneration in seedling-derived callus of jackfruit on semi-solid MS with 0.57 μM IAA and 8.87 μM BA and in callus derived from shoot tips of mature trees on semi-solid MS medium with 4.9 μM IBA, 0.54–53.71 μM NAA and 2.89 μM GA₃. Amin and Jaiswal (1993) reported regeneration of adventitious buds from compact callus derived from *in vitro* leaves of jackfruit on medium containing different concentrations of BA and NAA.

5.1.2. Somatic embryogenesis

Morus. Although Shajahan *et al.* (1995) reported induction of embryo-like structures in liquid cultures of *M. alba*, there have been no substantiated descriptions of somatic embryogenesis in *Moraceae*.

5.1.3. Haploid recovery

Morus. Regeneration of haploid plants would be useful in mulberry (dioecious species) for producing homozygous lines and to better understand its genetics (Jain *et al.*, 1996). Katagiri (1989) reported pollen division *in vitro*, and extensively studied the effects of sugars and alcohols on pollen culture (Katagiri and Modala, 1991). Haploid embryo differentiation (Seth *et al.*, 1992) and production of haploid plants from anthers was described by Shoukang *et al.* (1987). Globular masses and heart-shaped embryos were observed on semi-solid MS medium with 24.6–49.0 μM IBA and 22.19–66.57 μM BA. Embryos were mostly diploid, although

the microspore origin of the embryos was confirmed (Seth *et al.*, 1992). Jain *et al.* (1996) pre-treated anthers with kinetin and stored them at 4°C prior to culture of uninucleate microspores. Embryos were regenerated on medium supplemented with 2.69 µM NAA, 2.22 µM BA, 4.52 µM 2,4-dichlorophenoxy-acetic acid (2,4-D) and 600 mg/l PVP, and developed shoots were recovered. Jain *et al.* (1996) observed that the symmetrical division of microspores, the presence of an intact anther wall and the haploid nature of the induced roots were strong evidence of the regenerated haploid embryos.

Haploid mulberry plants have also been obtained from unpollinated ovary cultures (Thomas *et al.*, 1999; Bhojwani, 2001). Segments from *in vitro*-developed inflorescences were cultured on MS with 8.5 µM BA and 4.5 µM 2,4-D and, after transfer to MS with 4.5 µM 2,4-D, 6660 µM glycine and 1738 µM proline, produced gynogenic seedlings. After examination of 20 micro-propagated plants, 12 were observed to be haploid and eight were aneuploids.

5.1.4. Triploid plant recovery

Morus. Bhojwani (2001) reported that mulberry could be regenerated from cultured immature endosperm (3n) explants of cv. S-36. This is the only report of the recovery of triploids in this family.

5.1.5. Protoplast isolation and culture

Morus. *Broussonetia kazinoki* and *B. papyrifera* Vent. (paper mulberry) protoplasts have been isolated and plants have been regenerated (Oka and Ohyama, 1985, 1989; Katagiri, 1988, 1989, 1991). Tewary *et al.* (1995) reported microcolony formation from mesophyll protoplasts of *Morus* spp., but failed to obtain regeneration. Wei *et al.* (1994) reported plant regeneration from mesophyll protoplasts of *M. alba*. The yield of protoplasts obtained was approx. 2.5×10^7 /g and the proportion of intact protoplasts was as high as 96.8%. Tohjima *et al.* (1996) also isolated protoplasts but the yield was only 70%, and they were unable to regenerate plantlets.

5.2. Genetic manipulation

5.2.1. Mutation induction and somaclonal variation

Morus. Tewary *et al.* (2000) described a technique involving nodal segments for screening mulberry regenerates for tolerance of soil and osmotic stress. They supplemented semi-solid growth medium with 4.65 µM kinetin, 4.44 µM BA and 0.1–2.0% NaCl. A genotype, G3, was found to be salt tolerant under *ex vitro* conditions.

5.2.2. Genetic transformation

Morus. Although genetic transformation has been attempted with fruit species in the *Moraceae* family, achievements have been limited (Sugimura *et al.*, 2000). Low-efficiency leaf disc transformation of *M. alba* using *Agrobacterium tumefaciens* harbouring plasmid pBI121 containing the kanamycin resistance (neomycin phosphotransferase (*NPTII*)) and β-glucuronidase (*GUS*) genes was reported by Machii (1990). Transient expression of the *GUS* gene has been obtained via particle bombardment of callus, immature leaves and suspension cultures of *Morus* spp. (Machii *et al.*, 1996). Transformation via particle bombardment of Indian mulberry has been attempted (Bhatnagar *et al.*, 2002). Recently an effective and reproducible protocol for the production of transgenic plants has been reported for *M. indica* cv. K2 by *Agrobacterium*-mediated transformation (Bhatnagar and Khurana, 2003) using hypocotyls, cotyledons, leaf and leaf-derived callus. *Agrobacterium* strain LBA4404 was more effective than GV2260 and A281. The plasmid harbouring the *nptII* gene and *GUS* intron produced ten confirmed transformants as confirmed by Southern analysis. This protocol can now be utilized for stable transformation of mulberry with genes of desirable traits.

Agrobacterium rhizogenes-mediated transformation of hypocotyls of *M. indica* has been attempted (Oka and Tewary, 2000). Hair root cultures did not regenerate plants, but this could be useful for induction of rooting in difficult-to-root mulberry cultivars.

5.3. Germplasm preservation

Axillary buds and shoot tips of mulberry have been encapsulated for synthetic seed production (Pattnaik and Chand, 2000). Encapsulation of buds and subsequent regeneration of plants have been achieved for *M. indica*, *M. alba*, *M. australis*, *M. bombycis*, *M. cathayana*, *M. latifolia* and *M. nigra* (Bapat *et al.*, 1987; Machii, 1992b; Pattnaik and Chand, 2000). Synthetic seeds ensure greater viability and a higher survival rate of plant propagules (Rao and Bapat, 1992). According to Machii (1992b), adventitious buds are better for encapsulation than axillary buds, because of their potential to form multiple buds. An encapsulating matrix consisting of hormone-free MS nutrients or MS with BA is optimum for plantlet recovery. Bapat and Rao (1990) reported encapsulation of buds under non-sterile conditions by incorporating fungicides (50 mg/l Carbendazim, Benomyl and Bavastin) in the encapsulating matrix. Machii and Yamanouchi (1993) grew synthetic seeds of mulberry on simple substrates such as vermiculite, soil and sand. Pattnaik and Chand (2000) reported plantlet recovery from encapsulated buds directly on potting mixture moistened with either dilute MS basal medium or tap water. Refinement of the procedure is still necessary to extend the storage period and plantlet recovery from encapsulated buds. Synthetic seeds can be stored for specific intervals under refrigeration, thereby providing a means for short- and medium-term storage of germplasm.

Regeneration of mulberry plants from meristems stored in liquid nitrogen has been described (Yakuwa and Oak, 1988). The strategy involves stepwise slow freezing and storage of material in liquid nitrogen (Kantha and Engelmann, 1994). Addition of abscisic

acid (ABA) to the medium for better preservation of mulberry callus has been reported by Ohnishi *et al.* (1986). Sakai (1956) preserved mulberry twigs in two steps: (i) dehydration; and (ii) subsequent immersion in liquid nitrogen. Similar research with other members of the *Moraceae* has been limited (Niino and Sakai, 1993), although the embryo axes of jackfruit have been successfully cryopreserved (Thammasiri, 1999).

6. Conclusions

Breeding objectives among the members of the family *Moraceae* may not necessarily have a common goal. Improvement in leaf quality is a major emphasis in mulberry breeding, while fruit improvement is essential for jackfruit and fig. Micropropagation could be used to propagate superior trees in breeding programmes. *In vitro* selection has potential for use in screening material for biotic and abiotic stress tolerance (Gosal and Bajaj, 1984; Tewary *et al.*, 2000); however, better regeneration systems from cell cultures are needed for all species in the *Moraceae*. Improved *in vitro* systems are required for fig and the *Artocarpus* spp. before they can be genetically manipulated. Genetic transformation has potential for altering mulberry foliage to stimulate silk production. For jackfruit and breadfruit, improved fruit quality and extending the range of cultivation are important objectives that could be addressed. Fig improvement requires higher-yielding cultivars, improved fruit quality, nematode- and insect-resistant lines and elimination of caprification. Considering the importance of the seedless breadfruit as a staple crop, this species should be the focus of intensive research.

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13

Musaceae

Bananas and plantains (*Musa* spp. L.) are members of a monocotyledonous family of giant perennial herbs whose pseudostems are constituted of large leaf bases and which grow throughout the humid tropics and subtropics. There are three genera, *Ensete*, *Musa* and *Musella*, and 42 species in the family (Watson and Dallwitz, 1992 onwards). There are six *Ensete* species, which are spread throughout Asia and

Africa. Simmonds (1962) regarded *Ensete* as the remains of a primitive, contracting group of which small pockets of species, e.g. *E. homblei* from Zaire, are relics. *E. ventricosa* (Welw.) Cheesm. is a staple food crop in parts of southern Ethiopia at altitudes of 1500–3000 m. Edible starch is extracted from the corm and pseudostem, while fibre is made into cordage and sacking (Demeke, 1986).

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13.1 *Musa* spp. Banana and Plantain

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1. Introduction

1.1. Botany and history

Bananas and plantains have large pseudostems composed of tightly clasping leaf sheaths, slightly swollen at the base, and leaves which are spirally arranged with a large lamina and strong midrib. The bracts and flowers are inserted independently on the inflorescence axis. The bracts are usually deciduous and the basal flowers are generally female (Purseglove, 1972). Plants are rhizomatous and propagate vegetatively by means of suckers, although the wild species also propagate by seed.

The genus *Musa* consists of about 30 species that are found throughout South-east Asia from India to the Pacific Islands (Horry *et al.*, 1997). *M. ingens* has the distinction of being the largest herb in the world, with pseudostems up to 15 m tall and 2.5 m in circumference near the ground (Sharrock and Daniells, 1993). It is found in the tropical highlands of Papua New Guinea; however, the majority of *Musa* species are plants of the lowland tropics, requiring high temperatures, high relative humidity and high light intensities for optimal growth (Purseglove, 1972).

Modern banana cultivars arose as various inedible diploid subspecies of *M. acuminata*

(A genome), which crossed naturally, resulting in the production of numerous intra-specific hybrids. Some of these hybrids were parthenocarpic, female sterile and triploid in genomic structure and edible types would no doubt have been selected and maintained vegetatively by early farmers. The edible triploid bananas of South-east Asia were further selected according to vigour, fruit size and adaptability. Most of the bananas grown commercially today are essentially clones and variants of clones of these early selections.

As early farmers moved to drier monsoon areas of India, Myanmar, Thailand and the Philippines, the diploid and triploid *M. acuminata* cultivars hybridized with another species, *M. balbisiana* (B genome), to produce progeny with the genomes AB, AAB and ABB, the latter two representing the plantains. Studies by Shepherd and Ferreira (1982) and Carreel *et al.* (1994) have also shown that there are *Musa* cultivars that originated by hybridization of *M. acuminata* with *M. shizocarpa* (S genome) and *M. textilis* (T genome). It is thought that the subsequent dispersal of edible bananas and plantains outside Asia was effected solely by humans and the history of banana cultivation is therefore closely linked to the movement of human populations (Langdon, 1993; De Langhe and De Maret, 1999).

Bananas and plantains have achieved greater importance as cash or subsistence crops in regions away from their primary centres of origin. The export trade of dessert bananas, which consists of only a small number of AAA cultivars, developed in the mid-1900s in the Caribbean region and Central America with the advent of refrigerated shipping. It is only in recent years that the export of dessert bananas has gained prominence in Asian countries. In the case of plantains, 73% of the world crop is grown and consumed in West and Central Africa (FAO-STAT, 2000).

1.2. Importance

Bananas and plantains are arguably the world's most important fresh fruit. World production is estimated at 102,260,376 t annually (FAOSTAT, 2004), most of which is grown by smallholders for their own consumption and/or traded locally. World export trade, consisting essentially of the Cavendish dessert banana, is about 15 Mt and is worth US\$4.8 billion to exporting countries in Latin America, the Caribbean region and parts of Asia and Africa. Plantains are generally much starchier than bananas and can be eaten either ripe or unripe. Unripe fruits are usually boiled, fried or roasted. The *Musa* fruit has other uses, including beer making, edible dried chips, flour, etc. Male floral buds (distal male flowers with prominent bracts) are boiled as a vegetable, and leaves and pseudostems are used for thatching, fabric, cordage and mulch.

1.3. Breeding and genetics

Banana ($n = x = 11$) breeding has been considered a paradox. Most of the commercially important cultivars are parthenocarpic triploids and, by their nature, sterile. Indeed, seedlessness of the fruit is one characteristic that consumers demand and a trait that must be retained by the breeder. Despite these limitations, breeding is a viable option for the genetic improvement of *Musa*. One of the

first hybrids released from a breeding programme was FHIA-01, or 'Goldfinger' (AAAB), whose fruit was first sold commercially in the Republic of South Africa in 1992 (Whiley, 1996), 70 years after the first banana breeding programme commenced (Table 13.1.1).

The early banana breeding programmes were initiated in order to produce cultivars resistant to *Fusarium* wilt (*Fusarium oxysporum* f. sp. *cubense*) as a result of the imminent collapse of the industry, which was based on the susceptible 'Gros Michel' (AAA). These programmes were also based on the ability of 'Gros Michel' and its shorter natural mutants to produce seeds at a low frequency, e.g. there is an average of two seeds per bunch when hand pollinated with diploids (Simmonds, 1966). It was discovered that 'Gros Michel' does not undergo normal meiosis during sexual reproduction, but instead contributes an unreduced triploid gamete (Shepherd, 1974). Fertilization by pollen from a diploid yields tetraploids, many of which have been proven to be effectively seedless in cultivation. However, the wild, disease-resistant diploids contributed undesirable features to the tetraploid hybrids and none of the early 'Gros Michel'-like tetraploids were commercially released. Emphasis was given to the development of diploids with desirable agronomic qualities that could be crossed with disease-resistant accessions to provide diploid hybrids with combinations of agronomic excellence and disease resistance (Shepherd, 1974; Rowe, 1984). Unfortunately, the creation of *Fusarium* wilt- or black Sigatoka-resistant 'Gros Michel' and, later, 'Cavendish' (AAA) hybrids proved to be too difficult and it wasn't until the focus shifted to 'Pome', 'Sugar' and 'Plantain' (AAB) hybrids that the tetraploid breeding strategy proved to be successful.

Another breeding strategy, suggested by Vakili (1967) and further elaborated by Stover and Buddenhagen (1986), involves the induction of tetraploids from diploid stocks by colchicine treatment, selection for improved tetraploid lines and hybridization of those tetraploids with diploids to produce triploids for final selection. In this scheme

Table 13.1.1. Banana and plantain breeding programmes and their objectives.

Breeding programmes	Years	Breeding strategies	Breeding targets
ICTA – Trinidad – Jamaica	1922–1980 1924–1980	Tetraploid breeding; diploid improvement	Resistance to <i>Fusarium</i> wilt in 'Gros Michel' type
CBRS, Aduthurai – India	1949–	Tetraploid breeding; diploid improvement	Disease- and pest-resistant 'Pome', plantain and cooking banana types
United Fruit Company; FHIA – Honduras	1959–1984 1984–	Tetraploid breeding; diploid improvement	Resistance to <i>Fusarium</i> wilt, Sigatoka and burrowing nematodes in dessert bananas, plantains and cooking banana types
EMBRAPA–CNPMP – Brazil	1982–	Tetraploid breeding; diploid improvement	Resistance to <i>Fusarium</i> wilt and Sigatoka in 'Pome' and 'Sugar' types
CIRAD–FLHOR – Guadeloupe – Cameroon	1982– 1993–	Secondary triploid synthesis; diploid improvement	Resistance to Sigatoka and <i>Fusarium</i> wilt in plantain and dessert banana types
IITA – Nigeria – Uganda	1987– 1994–	Tetraploid breeding; diploid improvement; secondary triploid synthesis	High yield and resistance to Sigatoka in plantain types; resistance to <i>Fusarium</i> wilt and Sigatoka in highland beer and cooking bananas

ICTA, Imperial College of Tropical Agriculture; CBRS, Central Banana Research Station; FHIA, Fundación Hondureña de Investigación Agrícola; EMBRAPA–CNPMP, Empresa Brasileira de Pesquisa Agropecuária – Centro Nacional de Pesquisa de Mandioca e Fruticultura Tropical; CIRAD–FLHOR, Centre de Coopération Internationale en Recherche Agronomique pour le Développement – Département des Productions Fruitières et Horticoles; IITA, International Institute of Tropical Agriculture.

one is not limited to a single triploid parent that is susceptible to a number of pests and diseases. This triploid approach for *Musa* improvement has gained prominence in the Centre de Coopération Internationale en Recherche Agronomique pour le Développement – Département des Productions Fruitières et Horticoles (CIRAD–FLHOR) and International Institute of Tropical Agriculture (IITA) breeding programmes (Bakry and Horry, 1994; Ortiz and Vuylsteke, 1994).

Today, hybrids from the major breeding programmes are being evaluated in sites in many locations. This has been driven by the spread of more virulent forms of the fungal diseases black Sigatoka (*Mycosphaerella fijiensis*) and *Fusarium* wilt, particularly during the past 20 years. This prompted an increased international response towards the genetic improvement of *Musa* (Persley and

De Langhe, 1987). The programmes of the International Network for the Improvement of Banana and Plantain (INIBAP) have been instrumental in fostering the global testing and evaluation of *Musa* germplasm from both conventional and non-conventional breeding programmes (<http://www.inibap.org>). Plants are being assessed for resistance to black and yellow Sigatoka, *Fusarium* wilt and burrowing nematode, in addition to agronomic potential, in the testing countries.

2. Molecular Genetics

There is relatively little information available on either the banana genome or the molecular genetics of banana. This is not surprising as *Musa* has characteristics that do not lend themselves easily to genomic or genetic analysis. Bananas and plantains are large

plants, most > 2 m tall at flowering, and the majority of cultivars are essentially sterile. Conventional breeding programmes have only recently concentrated on providing information on the inheritance of valuable traits, and importantly there are very few characterized segregating populations that could be used for developing genetic and linkage maps. Despite these limitations, there has been significant information generated and this should increase dramatically in the near future due to international collaboration.

2.1. Banana genome

M. acuminata and *M. balbisiana* have haploid genomes ($n = x = 11$); however, *M. acuminata* has a slightly larger genome at an estimated 610 million bp compared with 560 million bp for *M. balbisiana* as determined by flow cytometry (Kumate *et al.*, 2001). Surprisingly, the *Musa* genome is only about three times the size of the *Arabidopsis* genome.

A number of important banana genomic resources are available or are in the process of being developed. The first banana bacterial artificial chromosome (BAC) library became available in 2000; this library was generated from the diploid *M. acuminata*, 'Calcutta 4'. While of no commercial value, 'Calcutta 4' is an important genetic resource as it has multiple disease resistances. This library was generated by GENefinder Genomic Resources at Texas A&M University (<http://hbz.tamu.edu>). The library, consisting of 23,040 clones and a genome coverage of 4.8 times, has an average insert size of 127 kb. Two other banana BAC libraries are currently being generated under the auspices of INIBAP through the Promusa programme (<http://www.inibap.org>). One of these libraries will be derived from a diploid *M. acuminata* and the other from a diploid *M. balbisiana*.

In parallel with the generation of BAC libraries, expressed sequence tag (EST) libraries have been developed or are being developed and, while the libraries themselves are not publicly available, a number of sequences from one of these libraries have

been deposited in databases. The most comprehensive EST libraries have been generated by Zeneca (now Syngenta) from 'Cavendish' (C. Bird, personal communication). The second group of EST libraries, at an earlier stage of development, is focused on the wild banana, *M. acuminata* ssp. *malaccensis*, a diploid fertile banana, which is resistant to a number of significant diseases (Othman *et al.*, 2000).

The amount of genomic information publicly available for *Musa* is very limited. This is easily demonstrated through searches of public databases. For instance, a search through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) nucleotide sequence database on 12 October 2001 resulted in 100,920 hits for *Sorghum* compared with 307 for *Musa*. Further, of the 307 hits, only 174 were actual *Musa* sequences and 157 of these were *M. acuminata* sequences. The *Musa* sequences consisted of 110 mRNA sequences, 68 repeat sequences or microsatellites, 23 gene sequences (including promoter sequences), seven ribosomal sequences and four virus-like or retrotransposon-like sequences.

A recent development in banana genomics has been the formation of the Global *Musa* Genomics Consortium, an international collaboration coordinated by INIBAP. While still in its formative stages, this a broad proposal which encompasses virtually all aspects of genomic analysis, including genome sequencing through to gene function, with particular emphasis on genes involved in disease resistance.

2.2. Markers and maps

A range of markers has been developed for *Musa* with particular applications in the study of diversity, genotyping, specific trait markers and potentially for tagging valuable genes. These include restriction fragment length polymorphisms (RFLPs), simple sequence repeats (SSRs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), sequence-tagged microsatellites (STMSs) and

methylation-sensitive amplification polymorphism (MSAP). RAPDs have often been used to assess genetic diversity and phylogenetic relationships within and between particular groups of bananas. For instance, Pillay *et al.* (2001) used RAPD markers to investigate the genetic diversity within 29 East African highland bananas that are all triploid *M. acuminata* (AAA). This specific group of bananas appeared to have a narrow genetic base, but they found sufficient genetic diversity to indicate that these RAPD markers could be used to distinguish between cultivars. Crouch *et al.* (2000) also found low genetic diversity among 76 plantain landraces using RAPD markers; importantly, however, they observed that there was a poor correlation between the genetic diversity estimated by RAPD markers compared with that based on a phenotype index. While both Crouch *et al.* (2000) and Pillay *et al.* (2001) have limited their investigations of genetic diversity to the banana and plantain cultivars and landraces of Africa, other scientists have taken a more global perspective and have analysed at least 160 different diploid bananas and 150 polyploid cultivars using RFLPs and STMs (Grapin and Lanaud, 1998; Grapin *et al.*, 1998; Carreel *et al.*, 2001; www.cirad.fr). The greatest genetic diversity was found within *M. acuminata*.

Micropropagation of bananas has become a standard practice for the production of banana planting material (see below), and markers for the early detection of somaclonal variants have been developed. Damasco *et al.* (1996) identified an RAPD marker that was specific for dwarfism in 'Williams' (AAA) and 'New Guinea Cavendish' (AAA). A single primer was developed such that a 1.5 kb band was amplified in all normal 'Cavendish' plants but was absent in all dwarf 'Cavendish' plants. This marker could be used for screening for the dwarf off-type of those cultivars *in vitro* (Damasco *et al.*, 1998). New techniques, such as representational difference analysis, suggest a molecular marker that is representative of a range of somaclonal variants and a range of cultivars may be developed (Cullis and Kunert, 2000). Peraza-Echeverria *et al.* (2001) developed

MSAP to compare the level of methylation in micropropagated bananas with that in conventionally propagated bananas to study somaclonal variation. The level of DNA methylation polymorphism in micropropagated bananas was between 3% and 1.7%, whereas no DNA methylation polymorphisms were detected in conventionally propagated bananas.

Perhaps the most important use of markers will be in the development of saturated maps of the banana genome. CIRAD has used isozymes, microsatellites, RFLPs, AFLPs and RAPDs to construct a composite map which covers 1227 cM and 11 linkage groups, confirming that the chromosome number is $n = x = 11$ (www.cirad.fr). One of the primary targets of the CIRAD mapping programme is to identify markers closely linked to disease resistance, particularly black Sigatoka.

2.3. Gene cloning

In parallel with the progress in mapping and markers, an increasing number of specific genes have been identified and characterized. Many of these have been associated with fruit and fruit ripening, e.g. pectate lyase genes (Pua *et al.*, 2001), banana fruit-specific lectin genes (Peumans *et al.*, 2000) and a uridine diphosphate (UDP) glucose pyrophosphorylase gene (Pua *et al.*, 2000). In contrast, Hermann *et al.* (2001a) isolated and characterized the expression of a banana actin gene that was constitutively expressed and was most similar to the vegetatively expressed plant actins. The promoter of this gene was also isolated, characterized and developed to drive transgene expression in bananas.

The major initial target for banana genomics is to identify and/or isolate disease resistance genes. This can either be approached using markers (as noted above) or through a comparative genomics approach. Specific resistance genes (R genes) have been identified in a number of crops. These R genes have been grouped into five classes based on their genomic structure. The most common of these R gene classes is class

3, which has a 5' leucine zipper domain or Toll-like sequence and a core nucleotide binding site (NBS) domain with a 3' leucine rich repeat (LRR) domain. In many other plant species, class 3 R gene analogues have been found using the polymerase chain reaction (PCR) with primers designed from conserved motifs within the NBS domain. These R gene analogues are usually present at very high frequency and appear to occur as multi-gene families (Crute and Pink, 1996). Taylor *et al.* (2000) have used a PCR strategy to identify class 3 R gene analogues in bananas and have characterized six R gene analogue families. All except one appear to be either single- or two-gene families, in contrast to the multi-gene families found in other plant species.

2.4. Banana streak badnavirus (BSV)

Perhaps the most surprising feature of the banana genome to date has been the discovery of the chromosomal integration of a plant virus. Banana streak badnavirus (BSV) has been known for some time as a significant but not devastating pathogen of bananas and is transmitted by mealybugs (Lockhart, 1994); however, its importance as a pathogen became significant after it was found that plants derived from tissue culture or the progeny of breeding programmes could sometimes have very high levels of virus infection even though they were derived from apparently virus-free plants/parents (Ndowora *et al.*, 1999; Hull *et al.*, 2000). It has recently been demonstrated that BSV sequences are integrated in the B genome. The integration pattern is complex, but, at least theoretically, complete episomal BSV sequences can be generated from these integrated sequences through recombination (Geering *et al.*, 2001). Importantly, while there is wide variation between episomal isolates of BSV in 'natural' infections, the episomal sequences from plants derived from apparently virus-free parents are almost identical to the integrated sequence, thus providing very strong evidence that the integrated sequences are recombined to produce infectious episomal BSV.

Chromosomal integration of plant viruses

is both interesting and important. The major banana breeding programmes have been severely impacted because it appears that the recombination/activation event is quite common during heterosis. Badnavirus-like sequences have been found in a wide range of *Musa* germplasm, including *M. acuminata* and *M. balbisiana*, but only *M. balbisiana* appears to have BSV sequences that can be recombined to produce episomal infection. As most banana breeding programmes use both genomes, the chance of infection in the progeny is high.

3. Micropropagation

There are more bananas being commercially micropropagated than any other fruit crop, with annual production figures estimated at >20 million propagules. Micropropagation was first applied to 'Cavendish' (AAA) by Ma and Shii (1972), who used shoot tips dissected from suckers as a way to rapidly multiply planting material free of *Fusarium* wilt. Micropropagation has since then been applied to a wide range of varieties (Vuylsteke, 1998), and that list continues to expand. Terminal floral axes have also been used to establish banana cultures (Cronauer and Krikorian, 1985). The first major field plantings were undertaken in Taiwan and Jamaica in the early 1980s (Hwang *et al.*, 1984; Ogelsby and Griffis, 1986).

3.1. Protocol

The procedures used for micropropagation of bananas and plantains have been reviewed in detail (Israeli *et al.*, 1995; Vuylsteke, 1998). Banana is commonly initiated into culture using the apical meristem contained in a 5 mm³ block of sterile tissue (Hamill *et al.*, 1993; Vuylsteke, 1998). Endogenous bacterial contamination may not always be eliminated by shoot tip culture. Persistent bacterial contamination is an obstacle to maintenance and distribution of banana germplasm *in vitro* and can hinder the establishment of suspension cultures, somatic embryogenesis and cryopreserva-

tion (Van den houwe *et al.*, 1998). An alternative method for establishing shoot cultures is meristem tip culture, which has been demonstrated to eliminate bacterial contamination from cultures initiated from suckers and *in vitro* or nursery plants (Van den houwe *et al.*, 1998; Hamill and Smith, 1999). Antibacterial agents, e.g. rifampicin, can be included in the establishment medium to control *Bacillus* infections.

The most widely used medium for micropropagation of banana is Murashige and Skoog (1962) medium (MS) containing 2–3% (w/v) sucrose, although Folliot and Marchal (1992) suggested 7–8% sucrose for optimizing growth. Other sugars, e.g. fructose and glucose (Marchal *et al.*, 1992), dextrose (De Guzman *et al.*, 1980) and mannitol (Bhat and Chandel, 1993), have also been utilized. Different banana genotypes can perform better on medium containing amended levels of micro- or macronutrients. In particular, potassium plays a significant role in nutrient uptake of *in vitro* plants (Zaidan *et al.* 1999a,b) and more vigorous plants may be produced when levels of potassium and/or phosphorus are increased (Marchal, 1990).

The most commonly used cytokinin for initiating shoot proliferation is benzyladenine (BA), usually at concentrations ranging from 10 to 25 μM . BA is superior to kinetin, N^6 -(2-isopentyl)adenine (2iP) and zeatin (Arinaitwe *et al.*, 2000). Usually, BA is the only plant growth regulator (Cronauer and Krikorian 1984a,b). Although roots are readily produced when all growth regulators are excluded, indolebutyric acid (IBA) has been used in medium to induce rooting (Vuylsteke, 1998). Rooting has also been induced with 1 μM indoleacetic acid (IAA), IBA and naphthaleneacetic acid (NAA) and with 0.1–0.25% activated charcoal.

Adenine sulphate has been included in culture medium for its synergistic effect with the cytokinins BA and kinetin (Hwang *et al.*, 1984; Vuylsteke, 1998). Other growth regulators, such as thidiazuron (TDZ) (Arinaitwe *et al.*, 2000), paclobutrazol (Wang and Duan, 1994; Wei *et al.*, 1999), choline chloride (Wei *et al.*, 1999), triazoles (Murali and Duncan, 1995) and ancymidol (Levin *et al.*, 1997), can improve multiplication and/or rooting.

Micropropagation strategies based on temporary immersion have the potential to increase production efficiencies. Alvard *et al.* (1993) compared different liquid culture methods with semi-solid medium and concluded that the highest weight gains and multiplication rates were achieved with aerated liquid treatments but that plants suffered hyperhydricity of the outer leaf sheaths. The use of bioreactors using banana stem clusters and shoot tips can also improve efficiency of banana micropropagation (Levin *et al.*, 1997; Ziv *et al.*, 1998).

During initiation of banana shoot tip cultures, there are often problems with blackening caused by oxidation of phenolics produced from the wounded tissue. The amount of blackening is genotype-dependent. Antioxidants, e.g. ascorbic acid (0.05–0.5 mM), citric acid (0.8 mM) and L-cysteine (0.02–0.3 mM), have been used to reduce this problem (Mante and Tepper, 1983; Vuylsteke and De Langhe, 1985). Activated charcoal (Bower and Fraser, 1982) has also been effective. Blackening is generally a problem only during the first few months of establishment of shoot tip cultures and does not cause as many problems in meristem culture provided the meristems are cultured in darkness for the first 5–7 days. Blackening can be managed by increasing subculture frequency to weekly intervals until cultures begin to multiply.

Genotypic differences account for variation in multiplication rate, vigour and rooting (Van den houwe *et al.*, 1995; Hirimburegama and Gamage, 1997). Variation can occur with suckers taken at the same time from the same mother plant (Smith and Hamill, 1991). Some genotypes are recalcitrant on standard media or will only multiply for a limited time (Hamill and Smith, 1999).

Banana tissue cultures grow well at 26–30°C (Vuylsteke, 1998) under cool, white commercial fluorescent light (50 $\mu\text{mol}/\text{m}^2/\text{s}$). A specific lighting regime does not appear to be essential, although some authors recommend increased light intensity (Cote *et al.*, 1990; Marchal *et al.*, 1992). Natural light has been used to replace or supplement artificial light (Kodym and Zapata-Arias, 2001).

Daniells and Smith (1991) described a

simple but effective procedure for plant establishment in the nursery. Sun-hardened, tissue-cultured banana plants establish well in the field and methods have been developed and reviewed by Robinson (1996) to optimize growth and yield. Generally plants produced from tissue culture need to be planted deeply, irrigated and de-suckered early to encourage development of productive plants and ratoon crops. Micropropagated banana plants can be more susceptible to various pests and diseases (De Waele *et al.*, 1998; Smith *et al.*, 1998). Infection can be overcome by inoculation of the rhizosphere or root system of micropropagated banana plants with beneficial bacteria, fungi or mycorrhizae (Lin and Chang, 1987; Declerck *et al.*, 1994; Jaizme-Vega *et al.*, 1998; Severn-Ellis, 1999; Pocasangre *et al.*, 2000; Smith *et al.*, 2001).

3.2. Accomplishments

Micropropagated banana plants generally outperform plants derived from conventional planting material with respect to yield, finger size, cycle time, emergence and crop uniformity, even into the ratoon crops (see Robinson, 1996, for review). When micropropagated plants have not provided higher yields than conventionally propagated material, this has been attributed to severe disease and suboptimal management (Vuylsteke and Ortiz, 1996). Micropropagated plants also produce significantly more suckers than conventionally propagated material (Drew and Smith, 1990; Israeli *et al.*, 1995), although this can be attributed to the physiological effect of growing plants in containers with a concomitantly larger root area at planting (Smith *et al.*, 2001). Lopez (1999) also suggests that micropropagated plantlets have a more efficient uptake of nutrients compared to suckers and this too can be attributed to a larger, more functional root system at the time of planting.

Many commercial banana-producing countries use tissue culture plants in an annual or biennial crop cycle to improve yield and reduce disease pressure (Israeli and Nameri, 1988) and in greenhouse production (Cabrera-Cabrera *et al.*, 1998).

Greenhouse production using tissue culture plants in the Canary Islands, Spain, has resulted in an increase in yield from 47.3 t/ha/year under conventional production systems to 83.7 t/ha/year (Galan-Sauco *et al.*, 1992). Micropropagated bananas not only have benefited major commercial producers but have had a positive impact on small landholders for subsistence agriculture (Kwa and Ganry, 1990; Sasson, 1997; Qaim, 2000). The adoption of micropropagated bananas in Kenya, for example, has resulted in greater disposable incomes to farmers, which bring added benefits for the entire community (Wambugu and Kiome, 2001).

Micropropagation of *Musa* has not been without obstacles. Somaclonal variation (Larkin and Scowcroft, 1981) has been widespread in micropropagated bananas and plantains (see below). Since the first commercial planting in the early 1980s, it has become apparent that banana micropropagation could result in high numbers of off-types, which are genotype-dependent (Smith, 1988; Vuylsteke *et al.*, 1991; Israeli *et al.*, 1995). Several distinct off-types have been described, including dwarfism in 'Cavendish' bananas (Smith, 1988; Israeli *et al.*, 1991; Cote *et al.*, 1993) and inflorescence changes in plantain (Vuylsteke *et al.*, 1991; Cote *et al.*, 1993). Low-vigour and low-production off-types are also common (Smith *et al.*, 1999). Apart from obvious visual characteristics such as distorted, mosaic or variegated plants, off-types cannot usually be identified *in vitro*.

The factors contributing to off-type production in banana tissue culture and their control have been reviewed (Smith, 1988; Cote *et al.*, 1993; Israeli *et al.*, 1995). Off-types can arise at any time in culture and they may arise from stimulation of adventitious buds (Damasco *et al.*, 1998); however, there is no evidence that growth regulators directly induce mutagenesis (Reuveni and Israeli, 1990). Nursery screening procedures have been developed for roguing off-types. 'Cavendish' (AAA) off-types can be identified on the basis of morphological characteristics (Israeli *et al.*, 1991; Smith and Hamill, 1993). Identification of molecular markers that can be used to screen for off-types is being attempted (Ford-Lloyd *et al.*, 1993;

Damasco *et al.*, 1996, 1998; Shoseyov *et al.*, 1998; Cullis and Kunert, 2000).

4. Virus Elimination

Shoot tip culture can be utilized to produce *Musa* plants free of certain pests and diseases, although banana viruses are readily transmitted via tissue culture (Thomas, 2000). Banana bunchy top nanovirus (BBTV) (Drew *et al.*, 1989, 1992; Wu and Su, 1991), cucumber mosaic cucumovirus (CMV) (Gupta, 1986), BSV (Krikorian *et al.*, 1999; Kubiriba *et al.*, 1999) and banana bract mosaic potyvirus (BBrMV) (Thomas and Magnaye, 1996) can all be transmitted via micropropagation. Thomas *et al.* (1995) showed that micropropagation eliminated BBTV from some, but not all, plantlets so that the virus was transmitted in an inconsistent way.

Meristem culture alone has been utilized to eliminate virus from banana (Gupta, 1986; Surga *et al.*, 1999), together with heat (Berg and Bustamante, 1974) or chemical treatments such as thiouracil (Bondok *et al.*, 1987); however, it is not possible to eliminate all viruses. *In vitro* conditions can trigger symptom expression due to activation of integrated DNA sequences of BSV (Kubiriba *et al.*, 1999; Ndowora *et al.*, 1999). There are established planting material schemes in many countries that ensure that micropropagated plants are virus-indexed and free from

fungal pathogens and pests (Magnaye *et al.*, 1995; Hamill, 2000; Hwang and Su, 2000). Certified disease-indexed bananas have ensured access to new cultivars across quarantine zones worldwide and have promoted the rapid introduction of elite selections (Vuylsteke, 1998).

5. Somatic Cell Genetics

5.1. Regeneration

5.1.1. Somatic embryogenesis

Somatic embryogenesis has been induced from a range of explants, including corm tissue (Novak *et al.*, 1989; Navarro *et al.*, 1997), leaf bases (Novak *et al.*, 1989), immature zygotic embryos (Escalant and Teisson, 1989), meristems (Dhed'a *et al.*, 1991; Ganapathi *et al.*, 2001) and immature male and female flowers (Shii *et al.*, 1992; Escalant *et al.*, 1994; Cote *et al.*, 1996; Grapin *et al.*, 1996, 2000; Becker, 1999; Ganapathi *et al.*, 1999). In order for these techniques to be useful as tools for genetic manipulation, they must be applicable to the important *Musa* cultivars and they must include a method for proliferation of the embryogenic cultures. Embryogenic cultures have been induced from meristems and immature flowers representing a wide range of genotypes (Table 13.1.2).

Table 13.1.2. Banana genotypes from which embryogenic suspension cultures have been generated from immature flower and meristem explants.

Explant	Genotype	Cultivar
Immature flowers	AA	SH3362, ² SF265, ¹ IRFA903 ¹
	AAA	Grand Nain, ^{2,3} Williams, ² Pei-Chiao, ⁹ Sien-Jen-Chiao, ⁹ Robusta, ⁹ B.f. ⁹
	AAAB	FHIA-01 ²
	AAB	Currare, ⁷ Currare Enano, ⁷ French Sombre ⁶
	ABB	Bluggoe ²
Meristems	AAA	Grand Nain, ⁸ Williams, ⁸ Cavendish 901, ⁸ Igitsiri, ⁸ Nakitengwa ⁸
	AB	Kamaramasenge, ⁸ Kisubi ⁸
	AAB	Three Hand Planty, ⁸ Bise Egome-1, ⁸ Agbagba, ⁸ Prata, ⁸ Lady Finger, ⁸ Rasthali ⁵
	ABB	Bluggoe, ^{4,8} Saba, ⁸ Cardaba ⁸

References: ¹Assani *et al.* (2001); ²Becker (1999); ³Cote *et al.* (1996); ⁴Dhed'a *et al.* (1991); ⁵Ganapathi *et al.* (2001); ⁶Grapin *et al.* (1996); ⁷Grapin *et al.* (2000); ⁸Schoofs (1997); ⁹Shii *et al.* (1992).

Induction. The induction of embryogenic cultures from immature male flowers was first described by Shii *et al.* (1992), and was refined by Escalant *et al.* (1994) and Cote *et al.* (1996). Its application was extended to the use of immature female flowers (Grapin *et al.*, 2000) and overcame the limitations for 'French Horn' (AAB) cultivars, which do not produce a viable male inflorescence. Clusters (hands) of immature flowers that are close to the apical meristem of the inflorescence are removed and cultured on induction medium (M1) consisting of MS medium, 18 μM 2,4-dichlorodiphenoxy-acetic acid (2,4-D), 5.4 μM NAA, 5.7 μM IAA and 4.1 μM biotin (Escalant *et al.*, 1994). The tissue is not subcultured until embryogenic cultures and somatic embryos appear. The embryogenic response can occur within a few weeks or can require 5 months. Another approach has involved induction of embryogenic cultures from proliferating axillary bud cultures (see below).

Maintenance. In order to establish embryogenic suspension cultures from meristems, a highly proliferating clump of meristems must initially be generated from *in vitro* shoot cultures on multiplication medium containing 1 μM IAA and, depending on the cultivar, varying concentrations of BA (Schoofs, 1997). After several subcultures on this medium, primordia gradually stop forming leaves and meristems enlarge, resulting in large white meristematic nodules. 'Bluggoe' (ABB) readily forms such 'cauliflower-like' meristematic clumps on medium with 10 μM BA. However, nine subcultures on 100 μM BA may be required for cultivars such as 'Cavendish' (AAA). Following the formation of meristem clumps, the upper 4–6 mm of these structures ('scalps') are used for induction of embryogenic cultures. Scalps are cultured on semi-solid medium, and proembryonic masses (PEMs) are removed and cultured in liquid medium (Schoofs, 1997) or inoculated directly into flasks containing liquid medium (Dhed'a *et al.*, 1991). Both semi-solid and liquid medium contain half-strength MS medium, 5 μM 2,4-D and 1 μM zeatin.

Flower-derived embryogenic cultures are transferred from induction medium to liquid medium (M2) consisting of MS medium,

4.5 μM 2,4-D and 4.1 μM biotin (Cote *et al.*, 1996). A suspension forms after 1–2 months, although regeneration capacity does not peak until about the fourth month. Embryogenic cultures have also been induced directly in liquid medium, as described by Dhed'a *et al.* (1991), and this appears to be highly effective (Becker *et al.*, 2000).

Embryogenic suspension cultures consist of a heterogeneous mixture of cell types (Schoofs, 1997; Georget *et al.*, 2000): (i) isolated cells; (ii) small cell aggregates about 50 to 100 μm diam.; and (iii) large cell clumps 200 μm to 2 mm diam. A description of these cell types can be found in Schoofs (1997) and Georget *et al.* (2000); cells are large, elongated and highly vacuolated, or rounded with starch reserves. Neither cell type is proembryonic, and they become necrotic. Cell aggregates (PEMs) consist of small cells with dense cytoplasm, which readily form somatic embryos. Large cell clumps, most of which can be removed by filtering during subculture or before plating, generally show low embryogenic potential. Thus, the regeneration capacity of individual cell lines is dependent on the composition of cell types.

Maturation. In order to stimulate embryo development from flower-derived cultures, PEMs are plated on filter paper discs over embryo development medium (M3) consisting of Schenk and Hildebrandt (SH) salts, MS vitamins, 1.1 μM NAA, 0.2 μM zeatin, 0.5 μM kinetin, 0.7 μM 2iP and 4.1 μM biotin (Cote *et al.*, 1996). Large numbers of somatic embryos can be generated from these cultures and Cote *et al.* (1996) reported an average of 3.7×10^5 embryos formed per 1 ml packed cell volume (PCV).

In the case of scalp-derived cultures, plants are recovered by plating PEMs on hormone-free semi-solid medium. After 3–4 weeks, immature somatic embryos are transferred on to medium containing 1 μM zeatin or BA for maturation. Somatic embryo germination has required higher levels of cytokinin (10 μM zeatin or BA). The regeneration potential of scalp-derived embryogenic suspension cultures appears to be similar to that of flower-derived cultures (Schoofs, 1997).

Germination and growth. After embryos from flower-derived cultures are removed from the filter paper and placed on germination medium (M4) consisting of MS salts, Morel and Wetmore (MW) vitamins, 1.1 μ M IAA and 0.2 μ M BAP (Cote *et al.*, 1996), the germination rate varies depending on their maturity. Cote *et al.* (1996) reported a germination rate of 3–20% for somatic embryos taken 80 days after plating cells on M3. Leaving somatic embryos on M3 for longer periods, e.g. up to 4 months, can increase the germination rate to >80% (Becker, 1999).

The high level of BA used to generate proliferating meristem cultures is a concern because of the potential for somaclonal variation; however, for 'Rasthali' (AAB), such proliferating meristem cultures are unnecessary for inducing embryogenic cultures. Ganapathi *et al.* (2001) plated longitudinal shoot tip sections on MS medium containing 4.1 μ M biotin, 9 μ M 2,4-D, 1 μ M zeatin and 2 g/l gellan gum. After 6 months, suspension cultures were initiated by transferring PEMs to M2 medium (Cote *et al.*, 1996).

Schoofs (1997) compared micropropagated banana to plants regenerated from scalp-derived embryogenic suspension cultures. With 'Williams' (AAA), 15% of micropropagated banana plants were off-types, whereas 18% of those derived from suspension cultures were variants. In this study only 110 'Williams' plants from embryogenic suspension cultures were observed for 6 months under glasshouse conditions. In a larger-scale experiment, Cote *et al.* (2000) measured the growth characteristics of 500 'Grand Nain' (AAA) plants regenerated from flower-derived embryogenic suspension cultures in the field through bunch set. Overall, there were no significant differences in growth characteristics. Some apparent off-types observed in the glasshouse displayed normal phenotypes after transfer to the field. Consequently, the frequency of off-types from embryogenic suspension culture-derived plants was nil while the frequency for those derived from shoot tip micropropagation was between 1.8% and 3.3%, depending on the genotype. These studies suggest that somaclonal variation in plants regenerated

from embryogenic suspension cultures is comparable to that of plants derived from shoot tip culture.

5.1.2. Protoplast isolation and culture

Megia *et al.* (1992) isolated protoplasts from suspension cells derived from immature seed of *Musa acuminata* ssp. *burmanica* 'Long Tavoy' (AA). Protoplasts were able to divide only in the presence of feeder cells. Using nurse cultures, Megia *et al.* (1993) and Panis *et al.* (1993) regenerated plants from protoplasts derived from embryogenic suspension cultures of 'Bluggoe' (ABB). When protoplasts were cultured at sufficient density (10^6 /ml), nurse cultures were not required for cell division (Panis *et al.*, 1993).

5.2. Genetic manipulation

5.2.1. Mutation induction and somaclonal variation

According to Predieri (2001), banana has benefited more from *in vitro*-induced mutation than any other fruit crop. This is due in no small part to the resources put into this approach by many institutions after considering the difficulties associated with breeding triploid cultivars. Mutations are heritable changes in the DNA sequence that are not derived from genetic segregation or recombination (Van Harten, 1998). Genetic variation can be induced by specific treatments with physical and chemical mutagens or can be a result of the tissue culture propagation of plants, e.g. somaclonal variation. Whether tissue culture induces mutations different from physical or chemical mutagens is still being debated (Larkin, 1998). The objective of induced mutations and somaclonal variation is to make single trait improvements in existing cultivars, e.g. disease resistance and agronomic traits under the control of one or a few genes, while otherwise retaining the original phenotype. According to Micke (1996), it is often forgotten that the rest of the genome is also exposed to the mutagenic effect of tissue culture/mutagens and also undergoes

genetic change. As a result many improvement programmes attempt to eliminate the unwanted off-types resulting from random mutations by subjecting the interesting mutants to further tissue culture passages and cycles of selection (Hwang *et al.*, 1993; Hwang, 2001).

Gamma-irradiation has been the physical mutagen of choice while ethyl methane-sulphonate (EMS) has been widely used as a chemical mutagen. Ploidy has also been manipulated chemically with colchicine and oryzalin to produce autotetraploids.

Physical mutagenesis. Although the use of physical mutagens in banana breeding was suggested as early as 1963 by Champion (Broertjes and Van Harten, 1988), only two banana cultivars have been released to date: 'Klue Hom Thong KU1' and 'Novaria' (Maluszynski, 2001). Both of these cultivars were obtained after gamma-irradiation of *in vitro* explants. Radiosensitivity studies of banana clones has demonstrated that some genotypes are more adversely affected than other cultivars (De Guzman *et al.*, 1980; Epp, 1987; Novak *et al.*, 1990; Smith *et al.*, 1994). Studies by Novak *et al.* (1990) have also shown that radiosensitivity varies with ploidy level. The median lethal dose (LD_{50}) was 20–25 Gy for the diploid (AA), 30–35 Gy for the triploid (AAA) and 35–40 Gy for the tetraploid (AAAA). Plantain triploid clones (AAB, ABB) had a radiosensitivity between 25 and 35 Gy. Within the population of plants that Novak *et al.* (1990) had regenerated from a 60 Gy-irradiated explant of 'Grande Naine' (AAA), an early-flowering plant was identified in the glasshouse. The GN-60A clone was micropropagated and plantlets were field-tested in Honduras, Australia, South Africa and Malaysia (Roux *et al.*, 1994). Independent trials confirmed that GN-60A flowered significantly earlier than 'Grande Naine' controls. The clone was subsequently released as 'Novaria' and entered commercial production in Malaysia in 1993 (Mak *et al.*, 1996).

Irradiation has also been used in conjunction with *in vitro* screening. Matsumoto and Yamaguchi (1990) selected an alu-

minium-tolerant mutant from irradiated protocorms of 'Cavendish' (AAA). Although gamma-irradiation has been attempted in order to produce AAA cultivars with resistance to *Fusarium* wilt (Epp, 1987; De Beer and Visser, 1994; Smith *et al.*, 1994; Bhagwat and Duncan, 1998a; Ho *et al.*, 2001) and greater tolerance has been demonstrated, none of the selections have been released to industry.

Chemical mutagenesis. Epp (1987) considered that EMS is more effective for producing variation than gamma-irradiation and treated meristems with 8–16 mM EMS for 4–5 days. Bhagwat and Duncan (1998b) recommended treating shoot apices with 200 mM EMS for 30 min and determined their dosage not by LD_{50} , which is often the case with mutagenic treatments, but on the survival rate, regeneration frequency and phenotypic variation among the regenerated shoots. Experiments with ^{14}C -EMS showed an effective role for dimethylsulphoxide (DMSO) as a carrier agent (Omar *et al.*, 1989). Other mutagens, e.g. sodium azide and diethylsulphate, have also been used for inducing variation from banana shoot apices (Bhagwat and Duncan, 1998b).

Chemical mutagens have also been used to manipulate ploidy in *Musa*, particularly for cross-breeding. Vakili (1967) found that tetraploidy could be readily induced with colchicine in *M. acuminata* and *M. balbisiana* seedlings and proposed a breeding scheme leading to resynthesis of triploids for final selection. Hamill *et al.* (1992) developed a reliable method for generating autotetraploids that involved treating shoot tips for 2 h in 1.25 mM colchicine solution to which 2% DMSO was added. In this way, improved diploids, with known traits such as disease resistance, could be treated *in vitro* and rapidly multiplied for breeding purposes or evaluation. Hamill *et al.* (1992) were able to induce autotetraploids in > 30% of shoot tips treated and a stable phenotype was eventually produced. Roux *et al.* (2001) demonstrated the importance of subculturing for dissociating chimeric tissues following mutagenesis. They treated 'Grande Naine' (AAA) with colchicine and found

that the proportion of chimerism was progressively reduced in the first three *in vitro* cycles, but even after six subcultures complete chimera dissociation was not achieved. Van Duren *et al.* (1996) have also shown that 15 μ M oryzalin and 2% DMSO, when applied to shoot tips for 7 days, were effective for producing high frequencies of tetraploids. These *in vitro* techniques for ploidy manipulation are now being effectively integrated into banana and plantain breeding programmes.

Somaclonal variation. Somaclonal variation has generated great interest as a potential source of novel and useful variability for *Musa* improvement. Hwang (2001) has successfully exploited somaclonal variation as a means of producing *Fusarium* wilt-resistant 'Cavendish' (AAA), and 12 resistant clones have been produced since 1985. One clone, 'Tai-Chiao No. 1', was released for commercial production in 1992; however, it has a more slender pseudostem and a longer growth cycle and produces a slightly smaller bunch than the industry standard. A dwarf variant (TC1-229) has now been described (Tang and Hwang, 1998) with improved agronomic characteristics while still retaining the disease resistance of the parent. These studies demonstrate that stepwise selection for beneficial traits using somaclonal variation can be incorporated in a banana improvement programme. This latter example also demonstrates the potential for exploiting variation in micropropagated plants produced for industry; TC1-229 was discovered on a farm following the commercial release of TC1.

Somaclonal variation from micropropagated plants has also been exploited with other banana and plantain cultivars (Cote *et al.*, 1993). Smith and Drew (1990) noted a variant of 'Mons Mari' (AAA) with fruit 2–3 cm larger than usual for all hands and with the potential to boost profitability through sales of extra large fruit. This clone has subsequently been released to industry as 'JD Special' (Daniells *et al.*, 1999). High-yielding plantain variants from 'Agbagba' (AAB) (Vuytsteke *et al.*, 1988) and 'Maricongo' (AAB) (Krikorian *et al.*, 1993)

have been identified and characterized. A variant from 'SH 3436' (AAAA) has been selected and was considered to have more resistance to Sigatoka diseases (Perez-Ponce and Orellana, 1994). A *Fusarium* wilt-tolerant variant of 'Pisang Rastali' (AAB) was discovered in a commercial plantation in Malaysia and was subsequently released as 'Mutiaru' (Ho *et al.*, 2001).

5.2.2. Somatic hybridization

Fusion of banana protoplasts from incompatible parents to create somatic hybrids is one method to circumvent the sterility barriers associated with many banana and plantain breeding programmes. Matsumoto and his collaborators (Matsumoto, 2001; Matsumoto *et al.*, 2002) have used electroporation to fuse protoplasts derived from embryogenic suspension cultures of commercial AAB cultivars with protoplasts derived from diploid floral bract tissue. Pentaploid hybrid cells, as confirmed by RAPD markers and flow cytometry, were produced. Using protoplast fusion of elite diploid clones, tetraploid plants could also be generated which, when crossed with other diploids, could reconstitute triploid clones (Assani *et al.*, 2001).

5.2.3. Genetic transformation

There has been great interest in the use of genetic transformation for introducing desirable genes into established cultivars, particularly 'Cavendish', and there is little chance of unintentional gene flow due to their extremely low fertility. These introduced genes may be derived from wild banana or from unrelated organisms. Efforts to genetically transform banana have largely focused on pest and disease resistance; however, there are some other interesting possibilities. Banana fruit have a relatively short shelf-life and prevention of premature ripening by minimizing exposure to ethylene is essential to deliver quality fruit to distant markets. One source of ethylene is the fruit itself; by knocking out genes associated with ethylene production, ripening can be delayed until ethylene is provided exogenously (Grierson, 1998). Another

possibility is to silence expression of polyphenol oxidase in the peel of fruit and thereby prevent unsightly blemishes associated with damage caused by pests or during harvesting and handling of fruit (Gooding *et al.*, 2001).

Direct DNA transfer via protoplast electroporation has been reported (Sagi *et al.*, 1994); however, a technically simpler and more efficient transformation method is microprojectile bombardment. Transgenic bananas have been generated by bombardment of embryogenic suspension cultures derived from immature flowers of 'Grand Nain' (AAA) (Becker *et al.*, 2000) and scalps of the cooking banana 'Bluggoe' (ABB) (Sagi *et al.*, 1995; Becker, 1999). Generally, these methods have involved harvesting cell suspensions during their rapid growth phase (4–6 days after subculture) and plating them on filter paper discs. Following bombardment, transformed cells are selected by transferring the discs to the appropriate medium. After somatic embryos have formed they are removed from the filter paper and placed in direct contact with the germination medium. Plants are regenerated 5–6 months after bombardment. With this method, the cultures are not disturbed after transformation so that somatic embryos that are derived from separate transformation events are distributed around the filter paper (Fig. 13.1.1). Also, a large number of transgenic lines can be generated using this method. An average of 25 transgenic 'Grand Nain' plants per bombarded plate (40 mg of cells per plate) can be produced (Becker, 1999), with Southern hybridization analysis demonstrating that these plants are derived from as many as eight independent transformation events.

Agrobacterium-mediated transformation has also been used to generate transgenic banana. May *et al.* (1995) wounded the meristems of *in vitro* plantlets by microprojectile bombardment prior to co-cultivation with *A. tumefaciens* (LBA4404 strain) on medium containing acetosyringone. Co-cultivated plantlets were subsequently subjected to several rounds of selection on multiplication medium to select for axillary buds derived from transformed sectors of

the plant. Due to problems with chimeras and low transformation rates, this technique is not commonly used; however, it did demonstrate that banana cells could be transformed using *Agrobacterium*. Subsequently, transgenic banana plants have been generated via *Agrobacterium*-mediated transformation of embryogenic suspension cultures. Ganapathi *et al.* (2001) incubated embryogenic suspension cultures of 'Rasthali' (ABB) with *A. tumefaciens* (EHA105 strain) in liquid medium supplemented with acetosyringone for 30 min prior to 3 days' co-cultivation on glass filter discs over semi-solid medium. Cells were subsequently selected for chlorosulfuron resistance and plants were regenerated on semi-solid medium. This technique was highly efficient, with 40 independently transformed plants being obtained in 4 months from 0.5 ml PCV of cell suspensions. Syngenta (formerly Zeneca Ltd) in the UK and DNA Plant Technologies Corporation in the USA have also used *Agrobacterium* and embryogenic suspension cultures to generate transgenic Cavendish (AAA) cultivars 'Grand Nain' and 'Williams' (Nisbet *et al.*, 2000).

Agents used to select transgenic banana include kanamycin and geneticin (resistance provided by neomycin phosphotransferase (*npt II*)) (May *et al.*, 1995; Becker, 1999), hygromycin (resistance provided by hygromycin B phosphotransferase (*hpt*)) (Sagi *et al.*, 1995) and chlorsulfuron (resistance provided by acetolactate synthase (*als*)) (Ganapathi *et al.*, 2001). Reporter genes used in banana include β -glucuronidase (*uidA* or GUS) (May *et al.*, 1995; Sagi *et al.*, 1995), the green fluorescent protein (GFP) (Dugdale *et al.*, 1998, 2000; Becker, 1999) and *npt II* (May *et al.*, 1995; Becker, 1999). GUS has been most commonly used because quantification is relatively simple; however, GFP is also useful. Dugdale *et al.* (2000) observed that GFP is more sensitive for visualizing activity of weak promoters. In some cases, promoters with no apparent activity when linked to GUS were shown to have low activity in vascular tissue when GFP was used as the reporter gene (Dugdale *et al.*, 2000). In addition, as the assay is non-destructive, GFP

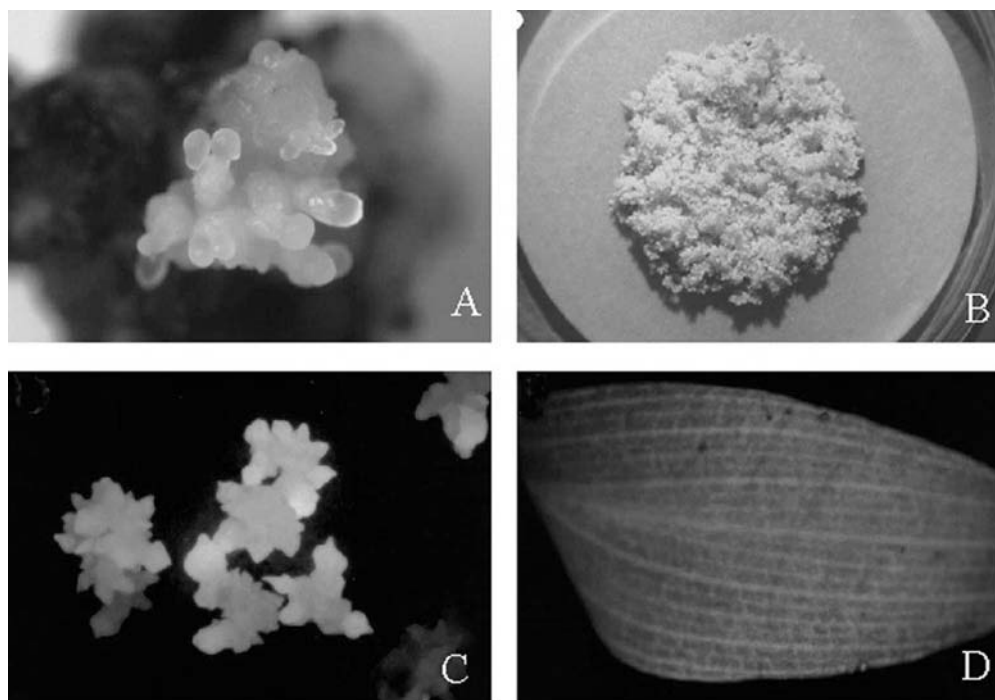


Fig. 13.1.1. Banana somatic embryogenesis and transformation. Flower hand of banana 'Grand Nain' 3 months after initiation on M1 medium (A). Somatic embryos have formed from white friable embryogenic culture, which is on the surface of yellow nodular callus. Most of the original explant has become necrotic. Somatic embryo formation derived from suspension cells on M3 medium with no antibiotic selection (B) and on selection medium after bombardment with a construct containing npt II and GFP (C). On selection, only regions of filter paper with stably transformed cells have produced somatic embryos which also fluoresce green when viewed under a fluorescence stereomicroscope (D).

can be visualized in transient assays as a measure of cell line transformation competence and during later stages as a measure of selection and regeneration efficiency (Fig. 13.1.1).

Different promoters have been shown to be active in banana, including those derived from viruses (Dugdale *et al.*, 1998; Schenk *et al.*, 1999, 2001), plant genes (Hermann *et al.*, 2001a) and *Agrobacterium* (May *et al.*, 1995; Ganapathi *et al.*, 2001). Techniques to enhance expression, such as the use of leader sequences (Sagi *et al.*, 1995) and introns (Dugdale *et al.*, 2000, 2001), have also been employed. Not all promoters have been thoroughly characterized with respect to their activity and tissue specificity in stably transformed banana plants. Those that have been characterized are listed in Table 13.1.3. There are several points to note about the data

currently published: (i) in stably transformed plants, the promoter derived from the maize polyubiquitin gene (Ubi-1) directs the highest activity in leaf tissue of all the promoters studied so far; (ii) promoter activity is much lower than with Ubi-1 unless enhancers such as the Ubi-1 intron are used (Dugdale *et al.*, 2001); and (iii) with the exception of cauliflower mosaic virus (CaMV) 35S, virus-derived promoters tend to direct higher activity in vascular and corm tissue and meristems.

Banana transformation has now progressed beyond development of transformation protocols to assessing the phenotype of plants transformed with potentially useful transgenes. Transgenic lines containing potential resistance genes to BBTV and BBrMV have been regenerated (Becker, 1999; Becker *et al.*, 2000). Plants were transformed

Table 13.1.3. Promoters whose activities have been characterized in stably transformed banana.

Promoter	Activity in leaf tissue ^a	Tissue specificity	Reference
CaMV 35S	0.171 (Ubi-1)	Constitutive	Becker (1999)
Ubi-1	5.7 (CaMV 35S)	Constitutive	Becker (1999)
Actin	NC	Constitutive	May <i>et al.</i> (1995)
BBTV DNA-2 (UI)	0.05 (CaMV 35S)	Vascular tissue, corm and meristems	Dugdale <i>et al.</i> (2000)
BBTV DNA-3 (UI)	0.01 (CaMV 35S)		
BBTV DNA-4 (UI)	1.45 (CaMV 35S)		
BBTV DNA-5 (UI)	1.57 (CaMV 35S)		
BBTV DNA-6 (UI)	0.87 (CaMV 35S)	Vascular tissue, corm and meristems	Dugdale <i>et al.</i> (2001)
S1 (UI)	~1 (Ubi-1)	Constitutive – higher activity in vascular tissue and meristems	Hermann <i>et al.</i> (2001b)
S2 (UI)	~1 (Ubi-1)		
ScBV	0.63 (CaMV 35S)	Constitutive – higher activity in corm and vascular tissue	Schenk <i>et al.</i> (1999)
Ba (UI)	0.72 (Ubi-1)	Constitutive	Hermann <i>et al.</i> (2001a)

^a Activity in leaf tissue as determined by fluorometric GUS assay. Number indicates the relative activity compared to either CaMV 35S or Ubi-1.

NC, no comparison can be made as plants were not transformed with other promoter constructs; UI, activity was enhanced using the maize polyubiquitin first exon and intron; CaMV 35S, cauliflower mosaic virus 35S; Ubi-1, maize polyubiquitin promoter with first exon and intron; Actin, rice actin promoter; BBTV, banana bunchy top virus, number indicating component; S1, S2, components associated with BBTV but not essential for viral infection; ScBV, sugarcane bacilliform virus; Ba, banana actin.

with genes important or essential for BBTV replication or with a gene encoding the coat protein of BBrMV. It is expected that in some cases these genes will be post-transcriptionally silenced. Therefore, when infected, there will be sequence-specific degradation of viral RNA (Dougherty *et al.*, 1994). Banana has also been transformed with genes encoding antimicrobial peptides (Sagi *et al.*, 1998). When extracts from the leaves of these plants were added to cultures of *M. fijiensis* (black Sigatoka), fungal growth was significantly decreased. Transgenic plants are currently being challenged with these diseases under glasshouse conditions.

Altered fruit characteristics have also been reported in transgenic banana. In field trials of transgenic banana, delayed fruit ripening using sense suppression of genes involved in ethylene biosynthesis has been described (Balint-Kurti *et al.*, 2001). While the above examples are only preliminary investigations into the regeneration of transgenic banana with useful new traits, it does indicate that transformation technology has advanced rapidly in the last few years and has the potential to contribute significantly to banana improvement.

5.3. Germplasm conservation and cryopreservation

Micropropagation has been an excellent mechanism for promoting banana conservation and germplasm distribution. Banana accessions can be initiated aseptically and stored in small culture rooms safe from the vagaries of weather, pest or disease. *In vitro* storage implies that plantlets can be disease-indexed and supplied on demand in a relatively short time frame.

Several methods have been used to reduce plant growth in tissue culture germplasm collections to overcome costs and reduce somaclonal variation. Increased osmotic potential of media using sucrose, ribose, glucose, fructose, lactose and mannitol has been shown to reduce growth rate (Ko *et al.*, 1991; Bhat and Chandel, 1993). A strategy that has been promoted as a solution to short-term storage and transportation of shoot tips has been encapsulation in sodium alginate (Rao *et al.*, 1993; Ganapathi *et al.*, 1998). To reduce growth of banana plants *in vitro*, Banerjee and De Langhe (1985) determined the minimum environmental growth conditions that allowed plants to survive. Their method is

now commonly used for medium-term storage and involves culturing plantlets at 16°C under low light intensity to reduce subculture frequency. Subculture frequencies can thereby be reduced to an average of 1 year, but genotype responses may vary from 3 to 22 months (Van den houwe *et al.*, 1995). The methods, benefits and obstacles to medium term *in vitro* storage of banana have been reviewed (Van den houwe, 1999). INIBAP maintains the world's largest *in vitro* banana collection (>1100 accessions) at the Katholieke Universiteit Leuven, Belgium; however, there are significant repositories in Australia, the Philippines and Taiwan.

Cryopreservation is effective for long-term storage of banana and plantain. Early research on banana cryopreservation was based on suspension cultures (Panis *et al.*, 1990). Embryogenic suspension cultures were stored in liquid nitrogen and somatic embryos were regenerated after 5 years in storage. Establishment of suspension cultures and somatic embryogenesis are highly genotype-dependent (Dhed'a *et al.*, 1991; Panis *et al.*, 1993). Banana meristems have also been cryopreserved, and two types of cultures have been utilized: (i) highly proliferating meristem cultures containing 'cauliflower-like' meristem clumps; and (ii) individual meristems isolated from micro-propagated plants. A practical manual for cryopreservation of *Musa* germplasm has recently been published that provides complete details of the procedures involved (Panis and Thinh, 2001).

Banana meristems are sensitive to osmotic stress and desiccation, which are both essential to preclude ice crystallization. Two methods have been developed for cryopreservation of banana meristem clumps for several genotypes (Panis *et al.*, 1996). Both procedures utilize highly proliferating cultures derived from meristems on medium containing up to 100 µM BA. Cryopreservation is also genotype-dependent. Some genotypes can survive following culture on high-sucrose pre-treatment medium followed by direct plunging into liquid nitrogen. Post-thaw survival rates of some genotypes can be improved by combining a simple sucrose pre-culture with vitrification

of the highly proliferating meristems prior to freezing in liquid nitrogen.

Thinh *et al.* (1999) have reported that vitrification could also be used for cryopreserving meristems (0.8–1 mm long) excised from rooted *in vitro* plants. A loading solution containing 2 M glycerol and 0.4 M sucrose enhances the tolerance of isolated meristems to dehydration by the vitrification solution.

6. Conclusions

Banana and plantain breeding has been supported by biotechnology since embryo rescue was first utilized for these crops (Cox *et al.*, 1960). Embryo culture was critical for banana breeding because few viable seeds are available from crosses involving edible cultivars. This technique has improved the recovery of plants from controlled crosses, often up to 50% (Stover and Simmonds, 1987).

Embryo culture and micropropagation are routinely used to recover and multiply material for crossing and for evaluation of hybrids. Somaclonal variation is also being exploited as a source of variability and for the generation of dwarf cultivars. The increased use of molecular markers is accelerating the process of recurrent selection of improved *Musa* germplasm, thereby facilitating the development of new hybrids. The future will witness the development of a new generation of 'Cavendish' cultivars produced by genetic transformation. There is also little doubt that the integration of the tools of biotechnology into banana and plantain genetic improvement programmes will enhance the sustainability of *Musa* production in producing countries of the developing world.

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14

Myrtaceae

The *Myrtaceae* family contains approx. 130 genera and 3000 species of trees and shrubs, mostly evergreen and distributed mainly in the tropics and subtropics (Watson and Dallwitz, 1992 onwards). The family contains many important species with edible fruits. Other than the guava, these include several *Syzygium* species, e.g. *S. aqueum*, *S. bracteatum*, *S. cerasoideum*, *S. cuminii*, *S. jambos*, *S. malaccense*, *S. samarangense* and *S. zeylanicum*, most of which are grown commercially on a small scale (Anon., 1976). *Feijoa sellowiana* Berg. (feijoa or the pineapple guava) bears fruits that resemble guava in size and appearance, and they can be consumed as dessert, in salad or cooked in a variety of

ways. *Myrciaria cauliflora* (jaboticaba) is often planted as an ornamental and the fruits are consumed fresh or made into jellies, wines and cordials (Hill, 1952). Many species within the *Myrtaceae* produce other valuable products: (i) essential oils and spices, e.g. clove *Eugenia caryophyllus*, allspice *Pimenta dioica*, bay leaf *Pimenta racemosa*; (ii) timber, tannins and firewood, e.g. *Eucalyptus* spp.; and (iii) sources of medicines, e.g. *Melaleuca leucadendron*, *S. cuminii*, *Psidium guajava* and *Barringtonia racemosa*. Some species are grown as ornamental trees (*Callistemon* spp.) and a few are used for land reclamation, e.g. *M. leucadendron* and *Leptospermum laevigatum* (Purseglove, 1968).

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<http://biodiversity.uno.edu/delta/>

14.1 *Psidium guajava* Guava

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1. Introduction

1.1. Botany and history

The guava is a shallow-rooted shrub or small tree 3–10 m in height with smooth, greenish or reddish brown bark, which peels off in thin flakes. The leaves are simple, opposite, 10–15 cm long, oval to oblong-elliptic, smooth and glandular. The flowers are perfect and epigynous and either solitary or in two- to three-flowered axillary cymes (2.5–3 cm diam.). The calyx is entire in the bud but splits into irregular four to six lobes (1–1.5 cm), which are reflexed, pubescent and persistent. There are four to five petals, white, obovate, concave, reflexed and 1–2 cm long. The stamens, which are numerous, are inserted in rows on a disc. The ovary is four- to five-locular, with a greenish yellow filiform style and capitate stigma. The fruit is a berry surmounted by calyx lobes and is globose, ovoid or pyriform, 4–10 cm diam. and 100–450 g. The exocarp is pale green to bright yellow and the mesocarp is fleshy, of varying thickness, white, yellow, red or pink with stone cells. The seeds are numerous, yellowish, bony and reniform with a curved embryo (Purseglove, 1968). The plant can be grown in a wide range of soil and climatic conditions. It is well adapted to variable rainfall and various soil pH levels (4.5–8.2)

and is tolerant of drought and salinity but susceptible to frost (Purseglove, 1968; Jaiswal and Amin, 1992).

The guava is indigenous to tropical America, where it occurs wild and under cultivation. In 1526, Oviedo reported that it was common in many parts of the West Indies and improved forms were planted by the local people. In the early 17th century, the Spaniards introduced guava from the neotropics into the Philippines across the Pacific and the Portuguese introduced it from the west to Africa and India. It has been widely dispersed by birds and has become naturalized throughout the tropics and subtropics (Purseglove, 1968).

1.2. Importance

World production statistics for guava are unavailable; however, the guava has major commercial importance in India, Egypt, South Africa, Brazil, Colombia and the Caribbean region (Wilson, 1980). The fruits are eaten fresh or as preserves and processed for use in dairy and baked products. The guava fruit is rich in vitamin C, carbohydrates, proteins, calcium, phosphorus, vitamin A, pantothenic acid, riboflavin, thiamine and niacin and is also a commercial source of pectin and oil (Wilson, 1980). Several other

Psidium species produce edible fruits, including *P. cattleianum*, *P. montanum*, *P. guineense*, *P. microphyllum* and *P. friedrichsthalianum* (Purseglove, 1968; Anon., 1969). International trade is for the most part limited to processed products.

1.3. Breeding and genetics

The genus *Psidium* ($2n = 2x = 22$) includes about 150 species, which are all fruit-bearing trees or shrubs. Although the common guava is diploid, there are naturally occurring as well as artificially produced triploid cultivars ($2n = 3x = 33$), which bear mostly seedless fruits (Purseglove, 1968; Jaiswal and Amin, 1992). Although a series of aneuploids has been artificially produced (Majumdar and Mukherjee, 1971, 1972), there is no report of higher polyploidy in guava. The floral structure limits the scope of breeding programmes for improvement of guava. Since guava is mostly self-pollinated and diploid, interspecific crosses for combining superior traits in a single genotype may be unsuccessful due to genome incompatibility (Jaiswal and Amin, 1992).

Guavas have traditionally been grown from seeds, although seedling trees are variable in both plant and fruit characteristics (Yadava, 1996a). Seedling trees begin to flower after 4–5 years. Vegetatively propagated trees begin to flower 2–3 years after establishment (Yadava, 1996a). Self and cross pollination occur and natural cross-pollination has been shown to be about 35% using the dominant red mesocarp as a marker (Purseglove, 1968).

1.3.1. Breeding objectives

Scions

Fruit quality. There is great diversity among cultivated guavas with respect to fruit size, bearing habit, yield, shape, quality and ripening season. Standards for selecting cultivars for processing include fruit diameter (at least 7.5 cm), fruit cavity diameter (no more than 3.75 cm), 200–300 g fresh weight

(FW), seed content (1–2%), dark pink colour, 9–12% soluble solids, vitamin C content, flesh with few stone cells and characteristic guava flavour (Soetopo, 1992). Guava fruit characteristics for fresh consumption include high yield, seedlessness, slow ripening and fruit firmness. In Hawaii, the USA and South Africa, dual purpose cultivars have been developed which bear fruits that are suitable for both processing and fresh consumption (Jaiswal and Amin, 1992). Some well-known cultivars include 'Lucknow 49', 'Allahabad Safeda', 'Chittidar', 'Nasik', 'Ka Hua Kula', 'Indonesian Seedless', 'Tathen White', 'Etheridge Selection', 'Oakey Pink', 'Beaumont', 'Ruby Supreme', etc. (Jaiswal and Amin, 1992; Yadava, 1996a,b). *P. guajava* closely resembles *P. guineense*, although the two species have not been hybridized (Anon., 1969).

Seedless cultivars tend to be shy bearers. To develop seedless cultivars with regular and more productive bearing habit, crosses have been made between 'Allahabad Safeda', a good-quality, heavy-bearing, seeded variety with a natural seedless triploid, shy-bearing selection (Majumdar and Mukherjee, 1971, 1972). Out of 73 seedling plants in the F_1 , 14 tetrasomic selections along with other aneuploids were promising, having fruits with normal shape and size and low seed number (Jaiswal and Amin, 1992). The 'Bangkok Golden Apple' is a cross between a Thai cultivar and 'Indonesian Seedless'. A positive influence of an aneuploid dwarfing guava rootstock (No. 82) on growth and productivity has been reported by Sharma *et al.* (1992). Morton (1987) reported a naturally occurring seedless triploid plant.

Rootstocks

Diseases. Guava trees are highly susceptible to wilt disease and to infection by soil-borne pathogens, including *Phytophthora* spp., which causes root rot disease. Three rootstocks with resistance to guava wilt disease were selected in South Africa by Schoeman and Vos (1998). These rootstocks were also recommended as scions based on yield and fruit quality (fruit flesh colour,

fruit mass, total soluble solids and breadth of fruit flesh). Vos *et al.* (1998) indicated that 25% of trees lost to the wilt disease in South Africa have been replanted with selections grafted on tolerant rootstocks. *P. friedrichsthalianum* (Chinese guava) is resistant to wilt disease and is compatible with guava. In nematode-affected areas, the Chinese guava has also demonstrated resistance to *Meloidogyne incognita*.

Dwarfing and yield. Little is known about the effects of rootstocks on vigour, cold tolerance, fruitfulness, fruit quality, mineral composition of leaves and disease tolerance (Mitra and Bose, 1985). Several species of *Psidium*, e.g. *P. cujavillis*, *P. molle*, *P. cattleianum* and *P. guineense*, can potentially be used as rootstocks for guava. The dwarfing effect of Chinese guava rootstock could be used in commercial plantings (Mitra and Bose, 1985). A dwarfing effect was also observed in trees grafted on *P. pumilum*. The sugar content of 'Allahabad Safeda' increased when grafted on to *P. pumilum* and, when grafted on to *P. cujavillis* rootstock, the ascorbic acid content was greater. The yield of 'Allahabad Safeda' increased on *P. cattleianum* rootstock (Mitra and Bose, 1985). Vasconcelos and Cardoso (1997) evaluated ten different guava cultivars as rootstock on the basis of growth characteristics, i.e. stem diameter, leaf area, plant height and leaf, stem and root dry weight. 'Riverside Vermelha' rootstock was shown to confer earliness, vigour and high growth rate.

2. Micropropagation

Regeneration of guava from shoot tip and nodal cultures of seedlings and from mature trees has been reported. Regeneration from shoot tip and nodal segments of 'Vietnamese Pear' seedling explants was described by Loh and Rao (1989). The optimum conditions included Murashige and Skoog (1962) (MS) medium supplemented with 0.4 μ M benzyladenine (BA). An average of 3.2 shoots per nodal segment was obtained after 8 weeks on medium with 2.2 μ M BA. Regenerated shoots rooted well (100%) in

MS basal medium. The protocols for rapid production of a large number of guava plants using explants from seedlings have also been described with other guava cultivars (Papadatou *et al.*, 1990; Mohamed-Yasseen *et al.*, 1995; Pontikis, 1996).

In vitro clonal propagation of some Indian cultivars of guava has been studied in detail by Amin (1987), Amin and Jaiswal (1987, 1988, 1989a,b) and Jaiswal and Amin (1987). Excessive exudation and oxidation of phenolic substances have been overcome with 0.5% polyvinylpyrrolidone (PVPP) and two or three changes of medium for the initial 10–15 days prior to culturing. Shading of orchard-grown plants (18 months old) has been reported to lower the phenolic compound accumulation with increased survival of apical meristem explants (from 0 to 40%) and of lateral bud explants (from 64 to 80%) (Leon De Sierralata *et al.*, 1997). Siddiqui and Farooq (1996) observed reduction in phenolic compound exudation and increased survival of explants by incorporating 250 mg/l PVPP in the culture medium. From nodal explants of mature trees of 'Banarasi' and 'Chittidar' cultivars, multiple shoot formation was initiated on semi-solid MS medium supplemented with 4.5 μ M BA alone or in combination with 0.6 μ M indoleacetic acid (IAA), 0.5 μ M indolebutyric acid (IBA) or 0.3 μ M gibberellic acid (GA_3). The optimum shoot multiplication rate (4.6 ± 1.3 shoots per culture) was obtained on medium with BA alone. By repeated subculture of *in vitro*-proliferated shoots, a shoot multiplication rate of three- to fourfold per subculture is possible. For optimum growth and proliferation of guava shoots *in vitro*, medium is supplemented accordingly: 30–45 g/l sucrose, 8 g/l agar at pH 5 (Amin and Jaiswal, 1989a).

Shoots can be rooted on half-strength MS semi-solid medium containing either 1.0 μ M IBA or 1.1 μ M NAA. Thirty-three per cent rooting frequency was observed in the initial cultures and 70–90% for shoots of the fifth and subsequent subcultures. Phloroglucinol (PG) either alone or with IBA or NAA inhibited *in vitro* rooting of guava microcuttings. Increasing the sucrose concentration from 15 to 45 g/l enhanced rooting (88–90%), although root length was unaffected. The

number of shoots that rooted was significantly higher at pH 5.5 and 6 than at lower pH values. There were more lateral roots in medium with pH 5.5 and 6 and they were more vigorous. Temperature also affected rooting; at 30°C 100% of the shoots produced roots and the induction and emergence of roots also occurred 7 days earlier than at 20 and 25°C (Amin and Jaiswal, 1989b). The regenerated plants can be successfully established in soil.

3. Somatic Cell Genetics

3.1. Regeneration

Somatic cell genetics is important for improving tropical and subtropical fruit trees and highly efficient *de novo* regeneration is a prerequisite (Litz and Jaiswal, 1991).

3.1.1. Organogenesis

Loh and Rao (1989) obtained shoot regeneration from cultured seedling hypocotyls of 'Vietnamese Pear' on MS medium with 0.04 μ M BA. They also described caulogenesis from leaves of proliferating shoot cultures derived from seedling explants (see Section 2).

3.1.2. Somatic embryogenesis

The advantages of somatic embryogenesis for efficient regeneration of some tropical fruit trees have been reviewed by Akhtar *et al.* (2000); however, this regeneration pathway has been described for relatively few myrtaceous fruit tree species. The effects of various factors that affect induction of embryogenic guava cultures and somatic embryo development, maturation and germination have been extensively studied (Akhtar and Jaiswal, 1995; Akhtar, 1997; Akhtar *et al.*, 2000).

Induction. Embryogenic cultures have been induced from immature zygotic embryos on MS medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with kinetin after 4–40 days (Jaiswal and

Akhtar, 1993; Akhtar, 1997). For optimum induction, zygotic embryos should be initially plated on full-strength semi-solid medium with 4.52 μ M 2,4-D alone or in combination with either 2.5 μ M IBA or 2.7 μ M NAA. Induction occurs from the hypocotyl and from within the embryonic axis. Histological preparations have demonstrated that somatic embryos develop directly, usually from subepidermal cells, and rarely from epidermal cells. Induction is temporally affected, and 8 days on induction medium resulted in the maximum number of cultures that produced somatic embryos.

The amount of embryogenic cultures, the number of responding embryos and the colour intensity of the cultures depended on the type and concentration of the auxin as well as length of exposure of zygotic embryo explants to the auxin. In the third week after explanting, cotyledonary somatic embryos are apparent in the embryogenic cultures, which are dark yellow, yellow-brown or light brown. Optimum induction conditions for 10-week-old zygotic embryo explants included medium with 10% sucrose and 4.5 μ M 2,4-D at $25 \pm 2^\circ\text{C}$. Other plant growth regulators, e.g. BA, kinetin, thidiazuron (TDZ), IBA and NAA, suppress induction (Akhtar, 1997; Akhtar *et al.*, 2000), although Ramirez Villalobos and Salazar Yamarte (1998) demonstrated that zeatin (0.5 μ M) was an effective induction agent. Light and dark treatments had no significant effects on the induction and development of somatic embryos. Akhtar (1997) observed that sugars other than sucrose were ineffective as a sole carbon source for induction. Glucose, maltose and lactose together with sucrose suppressed induction, whereas fructose, mannitol and sorbitol inhibited induction.

Maintenance. Recurrent somatic embryogenesis occurs in guava cultures (Akhtar and Jaiswal, 1995) and is dependent on developmental stages of somatic embryos in the cultures (Akhtar, 1997). Somatic embryos (8–14 weeks old) that develop after 8 days of exposure of zygotic embryo explants to 2,4-D are separated and subcultured on semi-solid MS medium without 2,4-D for induction of secondary embryogenesis. The cycle can be

carried out repetitively to produce secondary embryos. Somatic embryogenesis from torpedo stage embryos from 8-week-old cultures is enhanced with 4.5 μ M 2,4-D. Induction of recurrent embryogenesis is less in half-strength MS medium compared to full-strength medium. The cultures are maintained at $25 \pm 2^\circ\text{C}$ with a 16 h photoperiod.

Maturation. Development of somatic embryos is initiated by subculture from induction/maintenance medium to basal medium. The number of somatic embryos that develop from each zygotic embryo explant is variable, from one to > 1000. Development of somatic embryos is asynchronous (Fig. 14.1.1). Cotyledonary somatic embryo development requires 2–7 weeks on basal medium. Illumination (16 h photoperiod) during maturation is important for production of normal and convertible embryos. Somatic embryos that develop in darkness at low temperature are hyperhydric, milky white and physiologically immature compared to those that develop in darkness at 25°C , which possessed well-defined shoot and root meristems. The latter type of embryo converts to normal plantlets after transfer to germination medium under light conditions. Somatic embryos that develop at $30\text{--}35^\circ\text{C}$ become milky-white earlier than embryos at 25°C irrespective of the developmental stage, and convert into normal plantlets on germination medium. Somatic embryos from 6-week-old cultures germinate precociously after transfer to full- or half-strength MS basal medium without normal maturation, and form hyperhydric plants.

Germination. Different genotypes of guava have shown variation in frequency and efficiency of somatic embryo maturation and germination. Somatic embryos > 8 weeks old do not convert well, although plantlets are normal. Maximum plantlet conversion is obtained from 8-week-old somatic embryos. Elongated torpedo stage embryos (8 weeks old) convert into apparently normal plantlets at the highest frequency on half-strength MS basal medium. Semi-solid medium supports better germination than liquid medium. Benzyladenine, kinetin and

GA_3 do not improve germination of older embryos (Akhtar, 1997; Akhtar *et al.*, 2000). Growth of the plantlets for about 2 weeks on full-strength semi-solid MS basal medium with 3% sucrose is important prior to soil transfer and acclimatization (Akhtar *et al.*, 2000).

3.1.3. Haploid recovery

There has been little success with respect to recovery of haploid guava, although Babbar and Gupta (1986) observed early segmentation of guava microspores *in vitro* and demonstrated that a few globular embryos developed from these cultures. Callus developed from these structures on MS or Nitsch's medium with or without BA and 2,4-D, but the calluses could not be maintained.

3.2. Genetic manipulation

The related species *Feijoa sellowiana* has been genetically transformed (Oliveira *et al.*, 1996); however, regeneration of transformed plants was not achieved. There have been no reports of recovery of somaclonal variants, somatic hybrids or genetically transformed guava plants.

3.3. Germplasm preservation

Conditions for slow growth of guava in *in vitro* cultures have not been defined, and medium- and long-term storage have not been addressed. Jaiswal and Akhtar (1994) described a preliminary report of the encapsulation of guava somatic embryos and plantlet recovery.

4. Conclusions

The exploitation of *in vitro* methods is limited to a few fruit-bearing species of the *Myrtaceae*. Because of the short juvenile period of guava, this species could provide a unique model for the application of somatic cell genetic approaches for perennial fruit crop improvement. The areas in which such interventions

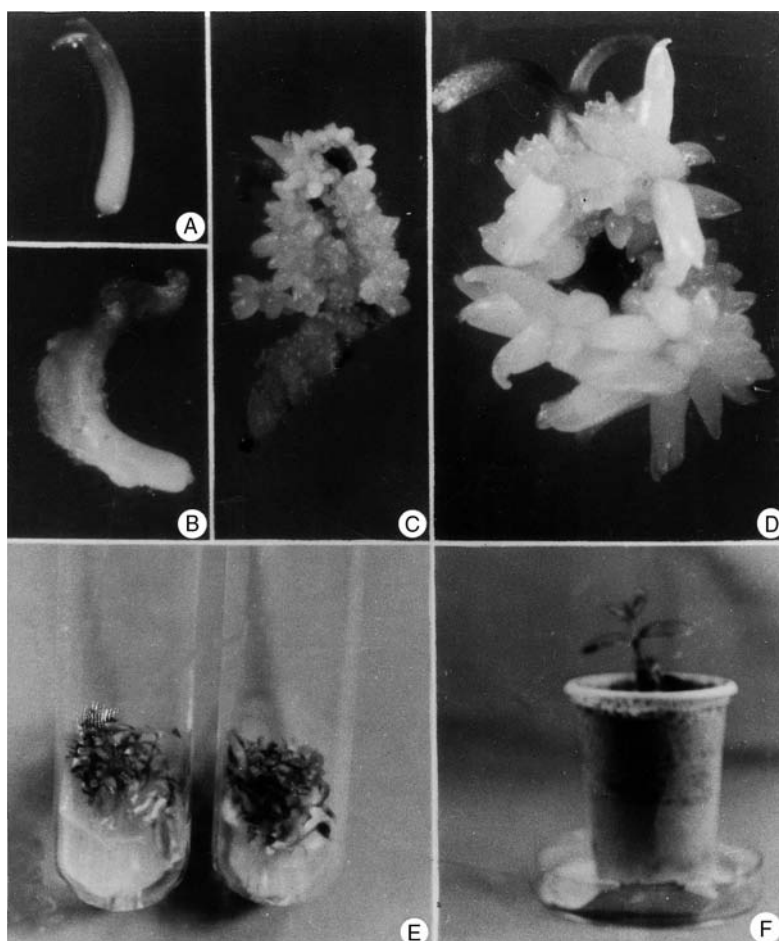


Fig. 14.1.1. Induction of somatic embryogenesis in zygotic embryos of guava. A. Zygotic embryo explant. $\times 9.0$. B. Zygotic embryo after 2 weeks on induction medium showing early development of embryogenic culture. $\times 9.0$. C. Early stages of somatic embryo development. $\times 9.0$. D. Torpedo stage somatic embryos. $\times 9.0$. E. Germinated somatic embryos. $\times 1.04$. F. Well-established plantlet after transplantation. $\times 0.47$.

are needed include development of fruits with longer shelf-life, seedlessness and resistance to root and fruit diseases. In recent years use of gene transfer technology has shown promis-

ing results in overcoming these problems in other crop species. Hence, future research on guava should focus on solving these problems using somatic cell genetic technologies.

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15

Oleaceae

The family *Oleaceae* includes approximately 25 genera and 900 species (Watson and Dallwitz, 1992 onwards) and is further subdivided into two subfamilies on the basis of chromosome number, i.e. *Jasminoidaceae* and *Oleideae*. In addition to olive, other well-known species in the family include the following: ash (*Fraxinus excelsior* L. and *F. angustifolia* Vahl.), privet (*Ligustrum vulgare* L.) and phyllirea (*Phyllirea angustifolia* L., *P. media* L. and *P. latifolia* L.). Other species of this family are cultivated as ornamental plants, including jasmine (*Jasminum fruticans* L.), lilac (*Syringa vulgaris* L.), *Fraxinus ornus* L., *Forsythia* × *intermedia* Zabel and *Osmanthus fragrans* Lour.

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15.1 *Olea europaea* Olive

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1. Introduction

1.1. Botany and history

The olive belongs to the *Olea* genus, which is included within the *Oleideae* subfamily and in the *Oleaceae* tribe. There are approximately 30–40 *Olea* species, which are distributed in Oceania, Asia, Africa and the Mediterranean region (Johnson, 1957). The genus is divided into two subgenera, *Olea* and *Paniculatae*; subgenus *Olea* is divided into two sections, *Olea* (including cultivated olive and its wild relatives) and *Ligustroides*. The section *Olea* includes the complex of *Olea europaea* L., the Mediterranean olive tree (Green and Wickens, 1989). These authors distinguished four subspecies according to their morphology and their geographical distribution: (i) *O. europaea* ssp. *europaea*, of the Mediterranean basin; (ii) *O. europaea* ssp. *laperrinei* (Batt. and Trab.) Ciferri, of the Sahara massifs; (iii) *O. europaea* ssp. *cerasiformis* (Webb. and Berth.) Kunk. and Sund., of the Canary Islands and Madeira; and (iv) *O. europaea* ssp. *cuspidata* (Wall. Ciferri), of Asia (China, India, Pakistan, Iran, south Arabia) and south-east Africa. *O. europaea* ssp. *europaea* includes the cultivated varieties (var. *europaea*) and wild plants (var. *sylvestris* = oleaster) of the Mediter-

anean basin. Green and Wickens (1989) and Zohary (1994) hypothesized that the different wild forms of the *O. europaea* complex have all contributed to the evolution of the cultivated olive.

Olive is considered to be a diploid species ($2n = 2x = 46$), but some tetraploid plants have been produced artificially (Rugini *et al.*, 1996). The 1C DNA content has been estimated for some cultivars to be approximately 2.2 pg (Rugini *et al.*, 1996), corresponding to a genome size of 2200 Mb (De la Rosa *et al.*, 2003).

Wild plants occur in the same areas as the domesticated olive, in the maquis and in uncultivated sites and show some morphological differences with cultivars, i.e. smaller fruit size and lower oil content in the mesocarp (Terral and Arnold-Simard 1996). Two distinct wild olives have been recognized: oleaster and feral forms. Oleaster occupies primary niches in undisturbed areas (Liphshitz *et al.*, 1991) as a constituent of evergreen plant associations. Numerous studies confirm that olive has been continuously present in the Mediterranean basin for several thousand years, particularly in the Middle East, long before its domestication. Palynological, anthropological and archaeological evidence (Carrion and Dupré, 1996; Watts *et al.*, 1996) demonstrates the presence of some forms of olive during the last glacia-

tion (18,000 BC) in the western and eastern Mediterranean regions. The discovery of oil-pressing tools has aided the dating of the beginning of oil extraction from olive. Olive was probably domesticated in the Middle East, north of the Dead Sea in the Jordan River valley c. 5700–5500 years BC (Zohary and Spiegel-Roy, 1975). According to Lipshchitz *et al.* (1991) olive domestication in this area occurred during the Bronze Age (5200 years BC). Due to their long life, there has been relatively little selection and the cultivated forms of olive are assumed to be very similar to their progenitors (Lipshchitz *et al.*, 1991). The spread of the cultivated olive to other countries of the Mediterranean region accompanied human migrations and involved different civilizations (Egyptian, Phoenician, Greek, Etruscan, Roman and Arab). The wild and cultivated varieties, representing two genetically separated complexes, have been interconnected as a result of occasional hybridizations, which could have allowed the introgression of genes from the wild forms into the cultivated varieties.

1.2. Importance

Olive is one of the most important tree crop species of the Mediterranean area and its global importance is rapidly increasing. Cultivation is approximately 9.8 million ha. The European Union (EU) is the main producer (78% of the olive oil production), and Spain and Italy are the most important producing countries. World olive oil production for 2001–2002 was approximately 2,688,500 t and that of canned olives about 1,426,000 t.

1.3. Breeding and genetics

There are > 1000 olive cultivars under cultivation. They have originated for the most part from selections made by growers over many centuries and have been propagated by rooting of cuttings and grafting. They have not been replaced by new high-performing genotypes, due to the low level of olive breeding.

1.3.1. Rootstocks

Major breeding objectives. Olives have been propagated mainly by grafting on to seedlings of scion cultivars or on wild rootstocks. The criteria for the selection of rootstocks include: (i) good rooting potential; (ii) control of scion vigour; (iii) resistance to drought, saline and heavy soils; (iv) resistance to root diseases; and (v) graft compatibility with the scion.

Breeding accomplishments. Very few studies have addressed the selection of clonal rootstocks. Preliminary work was concerned with the influence of rootstocks on scion performance. More recently, clonal rootstocks have been selected with high rooting ability and ability to control scion vigour (Baldoni and Fontanazza, 1990). Some rootstocks are able to control scion vigour and confer cold tolerance (Pannelli *et al.*, 2002).

1.3.2. Scions

Major breeding objectives. The primary goals of scion development include: (i) increased production (early bearing, high fruit production and weight and constant yield); (ii) resistance to pests, mainly the olive fruit fly (*Bactrocera oleae*); (iii) resistance to diseases, including leaf peacock spot (*Spilocaea oleagina*), *Verticillium* wilt (*Verticillium dahliae*) and olive knot (*Pseudomonas savastanoi*); (iv) tolerance of cold, drought and salinity stress; (v) adaptation to different microenvironmental conditions; and (vi) tolerance of heavy soils. Other important breeding objectives include: (vii) oil content and quality (fatty acid composition, polyphenol content, etc.); (viii) self-fertility (to avoid pollinators); and (ix) adaptation to intensive cultivation systems (dwarfing and adaptation to mechanical pruning and harvesting).

Breeding accomplishments. Olive breeding is complicated by its long juvenile period, which is genotype-dependent (Bellini, 1990; Fontanazza and Bartolozzi, 1998), but is typically > 10 years. The flowers of some olive

cultivars are fully male-sterile (Tombesi, 1978; Fontanazza, 1993; Bartolini and Guerriero, 1995), whereas others are self-fertile (Fontanazza *et al.*, 1990). Most, however, are partially self-fertile, which requires emasculation and is very tedious. Only a few cultivars have been developed by breeding. Lavee and his co-workers in Israel have released two cultivars, one for the table and one for oil extraction; the latter is also resistant to peach spot disease. Some characters, including vigour, leaf size and fruit shape, are dominant in F_1 progenies (Rugini and Lavee, 1992; Bellini *et al.*, 1995). The inheritance of other traits, such as carpological traits of fruit, flowering intensity, fruit set, period of fruit ripening and yield, is uncertain (Bellini, 1993; Parlati *et al.*, 1994; Bellini *et al.*, 1995). As an alternative to breeding, existing cultivar accessions are being screened for utility, although this approach has not been very successful (Morettini, 1972; Berenguer, 1978; Fontanazza, 1987). Many accessions having small tree size are under observation (Pannelli *et al.*, 1993).

2. Molecular Genetics

2.1. Gene cloning

The low percentage of oil (which rarely exceeds 25% of fresh weight), the peculiar composition of oleic acid (a C18:1 monounsaturated fatty acid), which is almost 75% of the total triacylglycerides, while linoleic acid is only 10%, and the quality parameters in virgin olive oils make the genetics of oil metabolism of special interest for the clarification of gene regulation involved in oil accumulation. Olive oil is one of the healthiest vegetable oils and efforts to improve other oil-producing crops have attempted to imitate its composition (Cahoon and Shanklin, 2000; Bruner *et al.*, 2001; Rahman *et al.*, 2001). Oil accumulation occurs in olive fruit mesocarp and to a lesser extent in the olive seed (Harwood and Sanchez, 2000).

Only a few genes have been isolated from olive (Table 15.1.1). Recently, genes encoding key enzymes in fatty acid biosynthesis, modification, triacylglycerol synthesis and storage

have been isolated. These are enoyl-acyl carrier protein (ACP) reductase (*ear*), stearoyl-ACP desaturase, Ω -6 plastidial desaturase (*fad6*), Ω -3 plastidial desaturase (*fad7*), cytochrome b5 (*cyt b5*), Ω -6 cytoplasmic desaturase (*fad2*), Ω -3 cytoplasmic desaturase (*fad3*), acyl coenzyme A (CoA), diacylglycerol acyltransferase (DGAT) and oleosin (P. Hatzopoulos, personal communication).

Stearoyl-ACP desaturase (Baldoni *et al.*, 1996) is the key enzyme for the conversion of saturated stearic acid (C18:0) to monounsaturated oleic acid (C18:1), the main component of olive oil and responsible for its high dietary value. Temporal and transient expression of the stearoyl-ACP desaturase gene has been studied during fruit development (Haralampidis *et al.*, 1998), and its expression is developmentally regulated with earlier expression in embryos than in mesocarp. A complementary DNA (cDNA) has been isolated encoding an Ω -3 fatty acid desaturase, which is responsible for the biosynthesis of a trienoic acid, linolenic acid, a major component of chloroplast membranes and a precursor of the oxylipins, e.g. methyl jasmonate. The latter are important in signal transduction pathways relating to plant development and responses to stress, and have been studied in leaves, anthers and embryos (Poghosyan *et al.*, 1999). Two cytochrome b5 genes and their spatial and temporal patterns of expression have been characterized during floral and fruit development (Martinkovskaya *et al.*, 1999). A triterpene synthase cDNA, cloned from olive leaves by polymerase chain reaction (PCR) amplification using primers designed from oxidosqualene cyclases, codes for the lupeol synthase protein (Shibuya *et al.*, 1999). The differential expression of DGAT genes has been evaluated in different olive tissues (Giannoulia *et al.*, 2000). The sequence of a partial cDNA clone (1402 bp) of the chloroplast ribulose 1,5-bisphosphate carboxylase large subunit (*rbcl*) gene of olive has been compared with that of other genera in order to establish the systematic position of the *Oleaceae* family (Wallander and Albert, 2000). Similarly, the chloroplast *ndhF* gene has been sequenced for phylogenetic studies (Olmstead *et al.*, 2000).

Table 15.1.1. Genes and expressed sequences currently identified in olive.

Gene encoding for	GenBank accession number	Authors	Year of publication	Length (base pairs)
18S ribosomal RNA gene	L49289	Johnson L.A., Soltis D.E., Soltis P.S.	Unpublished	1729 bp (partial sequence)
26S ribosomal RNA gene	AF479171	Soltis D.E., Senters A., Kim S., Thompson J., Soltis P.S., Zanis M., Ronse de Craene L., Endress P.K., Farris J.S	2003	1603 bp (partial sequence)
Acyl carrier protein (ACP)	AF428256	Guerrero C.M., Valpuesta V., Baldoni L.	Unpublished	763 bp
Acyl-CoA: diacylglycerol acyltransferase 1 (DGAT1)	AY445635	Giannoulia K., Hatzopoulos P.	Unpublished	1836 bp (complete cds)
Anthocyanidin synthase	AF384050	Ferrante A., Hunter D.A., Reid M.S.	Unpublished	789 bp (partial cds)
ATP synthase beta subunit	AJ236163	Albach D.C., Soltis P.S., Soltis D.E., Olmstead G.	Unpublished	1493 bp
Beta-actin (act1)	AF545569	Butowt R., Rodriguez-Garcia M.I.	Unpublished	408 bp (partial cds)
Chalcone synthase	AF384049	Ferrante A., Hunter D.A., Reid M.S.	Unpublished	571 bp (partial cds)
Chloroplast ribulose 1,5-bisphosphate carboxylase large subunit (rbcl) gene	AJ001766	Oxelmann B., Backlund M., Bremer B.	Unpublished	1402 bp (partial cds)
Chloroplast ribosomal protein S16	AJ431047	Bremer B., Bremer K., Heidari N., Erixon P., Olmstead R.G., Anderberg A.A., Kallersjo M., Barkhordarian E.	2002	881 bp
Copper/zinc superoxide dismutase (SOD1)	AF191342	Alche J.D., Castro A.J., Rodriguez-Garcia M.I.	Unpublished	276 bp (partial cds)
Cu/Zn superoxide dismutase	AJ428575	Butteroni C., Afferni C., Tinghino R., Barletta B., Iacovacci P., Orlandi A., Di Felice G., Pini C.	Unpublished	459 bp
Cu/Zn-superoxide dismutase	AF426829	Corpas F.J., Barroso J.B., Romero-Puertas M.C., Leon A.M., Valderrama R., del Rio L.A	Unpublished	312 bp (partial sequence)
Cycloartenol synthase	AB025344	Shibuya M., Zhang H., Endo A., Shishikura K., Kushihiro T., Ebizuka Y.	1999	1983 bp (partial cds)
Cytochrome b5 genes 1 and 2	AJ001369 AJ001370	Martsinkovskaya A.I., Poghosyan Z.P., Haralampidis K., Murphy D.J., Hatzopoulos P.	1997	688 bp 752 bp
Cytochrome c oxidase subunit I (cox1) mitochondrial gene	AF288707	Zilhao I.T., Tenreiro R.P., Fevereiro P.S	Unpublished	447 bp (partial cds)
Cytochrome oxidase; subunit 3, cox3 gene	Z70240 Z70241	Perrotta G., Cavallotti A., Quagliariello C.	1997	1817 bp 1818 bp
Enoyl ACP reductase (ear)	AY083164	Poghosyan Z., Hatzopoulos P.	Unpublished	1674 bp (complete cds)
Expansin	AF384051	Ferrante A., Hunter D.A., Reid M.S.	Unpublished	486 bp (partial cds)
Fatty acid desaturase 2 (fad2)	AY083163	Nikoloudakis N., Hatzopoulos P.	Unpublished	1452 bp (complete cds)
Hexose transporter pGIT	AY036055	Butowt R., Granot D., Rodriguez-Garcia M.I.	2003	2039 bp

Table 15.1.1. Continued.

Gene encoding for	GenBank accession number	Authors	Year of publication	Length (base pairs)
Iron superoxide dismutase-like	AY168776	Rodriguez-Serrano M., Romero-Puertas M.C., Valderrama R., Carreras A., Barroso J.B., del Rio L.A. Corpus F.J.	Unpublished	435 bp (partial sequence)
Lupeol synthase	AB025343	Shibuya M., Zhang H., Endo A., Shishikura K., Kushiro T., Ebizuka Y.	1999	2546 bp
Manganese superoxide dismutase	AF427107	Corpas F.J., Barroso J.B., Romero-Puertas M.C., Leon A., Valderrama R., del Rio L.A.	Unpublished	435 bp (partial cds)
Monosaccharide transporter (MST)	AF492010	Oliveira J.M., Geros H.V., Tavares R.M.	Unpublished	726 bp (partial cds)
NADH dehydrogenase (ndhF) chloroplast gene	AF027288	Oxelmann B., Backlund M., Bremer B.	Unpublished	2193 bp (partial cds)
NADH dehydrogenase subunit F (ndhF)	AF130163	Olmstead R.G., Kim K.J., Jansen R.K., Wagstaff S.J.	2000	2217 bp
Oleosin	AY083161	Giannoulia K., Haralampidis K., Milioni D. Hatzopoulos P.	Unpublished	792 bp (complete cds)
Photosystem II protein D1 (PSBA)	AY095446	Muleo R., Proietti C., Paolucci I., Harfouche A.L., Miano D.	Unpublished	942 bp (partial cds)
Polyubiquitin OUB1 and OUB2	AF429429 AF429430	Butowt R., Rodriguez-Garcia M.I.	Unpublished	1184 bp 1666 bp
Photosystem II protein D1 (PSBA)	AY095446	Muleo R., Proietti C., Paolucci I., Harfouche A.L., Miano D.	Unpublished	942 bp (partial cds)
Putative cullin protein	AY059387	Butowt R., Rodriguez-Garcia M.I.	Unpublished	2637 bp
Ribosomal protein S16 (rps16)	AF225275	Wallander E., Albert V.A.	2000	853 bp (partial intron)
RUB1 conjugated enzyme (ORCE)	AY157723	Butowt R., Rodriguez-Garcia M.I.	Unpublished	906 bp (complete cds)
Stearoyl-ACP desaturase	U58141	Baldoni L., Georgi L.L., Abbott A.G.	1996	1493 bp

2.2. Marker-assisted selection

For characters expressed at the mature phase, e.g. fruit traits, oil content and quality, tree architecture and size, it is necessary to wait for up to 15–18 years. It is possible to force seedling growth, obtaining early flowering and first fruit production 3–4 years after germination (Santos Antunes *et al.*, 1999). None the less, it would be more efficient to select for certain traits at the seedling stage if molecular markers linked to these traits were available. This is most effectively done by developing linkage genome maps, e.g. apple (Xu and Korban, 2000; see also Chapter 18.2), citrus (Sankar and Moore, 2001; see also Chapter 19.1) and peach (Lu *et al.*, 1999; see also Chapter 18.3). Molecular markers tightly linked to important traits have been identified in these species, e.g. scab resistance in apple (Xu *et al.*, 2001) and root-knot nematode resistance in cherry

(Lecouls *et al.*, 1999). Molecular markers linked to qualitative trait loci (QTLs) governing apomixis (Garcia *et al.*, 1999) or yield and seed number (Garcia *et al.*, 2000) have been identified in citrus, while QTLs controlling fruit quality have been mapped by Dirlwanger *et al.* (1999) in peach. Genome maps can be constructed from crossed progenies that are segregating for the characters of interest. Those markers co-segregating with the trait of interest are the tools for marker-assisted selection (MAS).

The first attempts to construct a linkage genome map for olive have been reported (Baldoni *et al.*, 1999; De la Rosa *et al.*, 2000, 2003) using the progeny derived from the cross between two highly heterozygous cultivars as a segregating population. The use of an F_1 progeny, allowing the use of markers in the condition of a pseudo-test cross, limits the number of useful markers and results in the construction of two distinct maps: one for each

parental line. This can be overcome by the use of markers that produce a very high number of scorable markers in each reaction, e.g. amplified fragment length polymorphisms (AFLPs), and by the use of co-dominant markers, i.e. microsatellites, to anchor the different maps in a general one for the species.

In the 'Leccino' map, 249 markers (110 random amplified polymorphic DNAs (RAPDs), 127 AFLPs, eight restrictive fragment length polymorphisms (RFLPs) and three simple sequence repeats (SSRs)) were linked. This resulted in 22 major linkage groups and 17 minor groups with fewer than four markers. In the 'Dolce Agogia' map, 236 markers (93 RAPDs, 133 AFLPs, six RFLPs and four SSRs) were linked. In this case, 27 major linkage groups and three minor groups were obtained.

2.3. Molecular markers

There is great genetic diversity within cultivars and wild populations of the species in the Mediterranean region. This germplasm represents an important source of variability for future breeding. To exploit this resource, more information is needed, including: (i) the level and distribution of variability within cultivars; (ii) the genetic relationships among cultivars and with wild olives; (iii) markers linked to the main traits under selection; and (iv) characterization of genes controlling these characters and their regulation and expression at different developmental phases and in different tissues.

Until recently, the variability within *O. europaea* germplasm has been restricted to morphological descriptors. Biochemical and molecular analyses have been carried out using isozymes (Ouazzani *et al.*, 1993, 1996; Trujillo and Rallo, 1995) and RAPDs (Fabbri *et al.*, 1995), primarily for cultivar identification. Most recently, a range of DNA markers have been utilized to study the olive genome with the following objectives: (i) revision of the taxonomy within the genus; (ii) analysis of the relationships between cultivated and wild olives; (iii) determining the origin of cultivars under cultivation; (iv) characterization and identification of cultivars; and (v)

olive genome mapping and the identification of markers linked to the most important agronomic traits.

2.3.1. Taxonomic revision

The geographic distribution of variability within the *Olea* genus and genetic relationships among the different species have been studied using different molecular markers. The chloroplast DNA (cpDNA) RFLP, performed on 15 *Olea* taxa, has resulted in the clear separation of species in section *Olea* from those of section *Ligustroides*; the latter group of species appear to possess ancestral variants of cpDNA (Lumaret *et al.*, 2000). AFLP analysis has been used to distinguish *Olea* species from the Indian Ocean and Oceania from the species of East Africa and Asia, which clustered together. *Olea* spp. from north-west Africa, e.g. *O. laperrinei* and *O. maroccana*, showed a higher level of similarity with cultivated and wild samples of *O. europaea* (Angiolillo *et al.*, 1999). Analyses performed on ribosomal DNA (rDNA), cpDNA and mitochondrial DNA (mtDNA) polymorphisms have confirmed the ancient separation of the genus *Olea* into two subgenera: Asia–Australia (subgenus *Paniculatae*) and Africa (subgenus *Olea*). Three distinct main phyla were characterized: *O. cuspidata* – *O. chrysophylla* (= *cuspidata* phylum) of Asia, *O. africana* (= *africana* phylum) East and South Africa, and *O. europaea* – *O. laperrinei* – *O. maroccana* – *O. cerasiformis* (= *europaea* phylum) of the Mediterranean region, Sahara and north-west Africa (Besnard and Bervillé, 2002; Besnard *et al.*, 2002a,b). According to cpDNA and mtDNA analyses, *O. europaea* ssp. *maroccana* represents a well-differentiated and relict taxon, belonging to the same subtaxon as *O. europaea* ssp. *guanchica* (Medail *et al.*, 2001).

The phylogenetic relationships within the *Olea* genus have been assessed by nucleotide variation at a non-coding cpDNA region in some *Olea* species. In this manner, four groups could be distinguished: species *O. capensis* and *O. lancea*, both belonging to subgenus *Ligustroides*, the *Olea* forms from south-east Africa, those from Asia and the taxa of north-west Africa and the Mediterranean region, which include the

cultivated olive (Baldoni *et al.*, 2002). These relationships are consistent with those previously reported with cpDNA RFLPs (Lumaret *et al.*, 2000).

Two tandemly repeated sequences (81-bp and pOS218) were isolated by Katsiotis *et al.* (1998) and were localized by *in situ* hybridization on the chromosomes, showing a telomeric or interstitial location. These elements are also present in oleasters and in *O. chrysophylla* and *O. africana*, but are absent in other genera of the *Oleaceae*, i.e. *Phyllirea*, *Forsythia*, *Ligustrum*, *Parasyringa* and *Jasminum*. The amount and organization of heterochromatin and the frequency of other tandem repeated sequences (*OeTaq80*, *OeTaq178*, *OeGEM86*) indicate that DNA content is positively correlated with the copy number of DNA repeats in the genomes of different *Olea* forms (Bitonti *et al.*, 1999; Lorite *et al.*, 2001; Contento *et al.*, 2002). Cytological hybridization of these tandem repeats has made it possible to distinguish most of the olive chromosomes and to reveal structural heterozygosity in three chromosome pairs (Minelli *et al.*, 2000).

2.3.2. Genetic relationships among cultivated and wild olives

The wild relatives of cultivated species contain traits, e.g. biotic stress resistance, adaptation to extreme environmental conditions, plant vigour and architecture, that could be introgressed into cultivars by conventional breeding or by gene transfer. RFLP markers have been identified from mitochondrial, chloroplast and nuclear DNA which, with isozyme markers, provide evidence for the survival of indigenous oleaster populations, particularly in the western part of the Mediterranean region. The domesticated olive represents a subset of the genetic variation in genuinely wild olive populations that persist today (Lumaret and Ouazzani, 2001). Five distinct chlorotypes were distinguished on the basis of cpDNA restriction patterns, and wild olives were more variable than cultivars (Amane *et al.*, 1999). With mtDNA RFLPs, different mitotypes have been identified. Within wild populations, a clear distinction exists between the eastern and western Mediterranean regions. For cultivars, a more complex mitotype distri-

bution exists, probably due to exchanges of plant material during the long history of olive cultivation (Besnard and Bervillé, 2000; Bronzini de Caraffa *et al.*, 2002a,b). The two different forms of wild olives (oleasters and feral forms) have been distinguished by isozymes (Lumaret *et al.*, 1997) and with AFLP markers (Angiolillo *et al.*, 1999).

The earliest attempts to clarify olive domestication involved the use of isozymes (Loukas and Krimbas, 1983), and ancient cultivars could be distinguished from more recently selections. The colonization history of *O. europaea* has been analysed based on internal transcribed spacer 1 (ITS-1) sequences, RAPDs and inter-simple sequence repeats (ISSRs) in Macronesia (Hess *et al.*, 2000). *O. europaea* retroelements have also been identified (Hernandez *et al.*, 2001a) and the total copy number in the genome has been estimated (Stergiou *et al.*, 2002).

2.3.3. Cultivar identification

Molecular markers can be used for cultivar identification, to determine the origin of cultivars and for detection of fraud in olive oil marketing. Isozymes were first used for cultivar identification (Pontikis *et al.*, 1980; Trujillo and Rallo, 1995). More recently, cultivar identification has been based on DNA markers, e.g. RAPDs (Bogani *et al.*, 1994; Fabbri *et al.*, 1995; Cresti *et al.*, 1996; Wiesman *et al.*, 1998), using different protocols (Belaj *et al.*, 1999, 2001, 2002; Mekuria *et al.*, 1999; Barranco *et al.*, 2000; Gemas *et al.*, 2000; Besnard *et al.*, 2001b). More than 200 cultivars in the World Olive Germplasm Bank (Cordoba, Spain) have been characterized using relatively few primers. Cultivars with a common area of origin show a high similarity. Cultivars cultivated in distant areas often show close similarity, which supports the hypothesis of a multilocal selection for most cultivars (Besnard *et al.*, 2001a). AFLP data are available on the cultivars of most of the Italian regions (Baldoni *et al.*, 2000). For cultivar characterization, SSRs (Rallo *et al.*, 2000; Sefc *et al.*, 2000; Carriero *et al.*, 2002; Cipriani *et al.*, 2002), as well as ISSRs (Pasqualone *et al.*, 2001; Vargas and Kadereit, 2001), have been used. Development of sequence-

characterized amplified region (SCAR) markers has also been attempted from RAPDs (Hernandez *et al.*, 2001b). Besnard *et al.* (2000) used RFLPs to analyse mtDNA and cpDNA of a progeny derived from the cross of male-sterile 'Olivière' × 'Arbequina', and established that male sterility is maternally inherited and is associated with the chlorotype CCK and mitotype MCK.

3. Micropropagation

Micropropagation of olive by shoot tip and nodal culture has made few advances: (i) some important olive cultivars, e.g. 'Frantoio', 'Kalamata', 'Leccino', 'Pendolino' and 'Picholine', are difficult to establish *in vitro* (Zuccherelli and Zuccherelli, 2000); and (ii) the stimulation of axillary buds and subsequent shoot elongation require high concentrations of zeatin, which is relatively expensive and contributes to high production cost. None the less, olive micropropagation is starting to become competitive for some cultivars, and may even be more convenient than traditional propagation by cuttings (Rugini *et al.*, 2001). Olive micropropagation has been reviewed (Rugini and Fedeli, 1990; Cañas *et al.*, 1992; Lambardi and Rugini, 2003). Early studies focused on the use of axillary bud development from embryos and seedlings (Bao *et al.*, 1980; García-Berenguer and Durán González, 1990; Cañas *et al.*, 1992). Adult trees pose a number of problems, including oxidation of tissues from field-grown or greenhouse-grown plants, microbial contamination and unresponsive cultivars.

Successful establishment of shoot tip and nodal explants is related to vigorously growing (woody or semiherbaceous) young stock plants. Forcing lateral basal buds of the new shoots when they are still attached to the woody nodal explant and eliminating the apex at each subculture for several subcultures have been effective (Rugini *et al.*, 2000). Micrografting has potential as a pretreatment for rejuvenating elite material for micropropagation. Revilla *et al.* (1996) attempted to rejuvenate mature olive trees ('Arbequina') by serial micrografting. Troncoso *et al.* (1999) cleft-micrografted uninodal explants (from *in*

vitro-grown seedlings of 'Cañivano') on *in vitro*-grown 'Arbequina' seedlings.

The medium for initiating cultures can be used for woody and herbaceous nodal explants (Rugini, 1984) and for shoot tips (Martino *et al.*, 1999) of elite 'Moraiolo'. Since shoot tips and apical buds are not good starting material, nodal explants are used, and are collected in winter or early spring from field-grown plants or from a greenhouse. Decontaminated nodes are explanted on medium containing a low concentration (mg/l) of macroelements: KNO₃ 500, NH₄NO₃ 100, CaCl₂ 2H₂O 40, KH₂PO₄ 50, MgSO₄ 7H₂O 250, FeSO₄ 13.9, sodium ethylenediamine tetra-acetic acid (NaEDTA) 18.6; quarter-strength Murashige and Skoog (1962) (MS) microelements, half-strength MS vitamins, sucrose 2%, inositol 50, zeatin riboside 0.5, thiamine 10 and 0.6% agar.

Shoots that develop from primary explants normally show strong apical dominance and initially, except for few genotypes, grow stentately. For less responsive cultivars, Rugini *et al.* (2000) suggested that lateral basal buds of new shoots could be forced when they are still attached to the explant by decapitating the apex of each shoot at each subculture for several subcultures. Proliferation medium (OM) was formulated on the basis of an analysis of the main mineral elements found in shoot apices of vigorous field-grown plants and in mature zygotic embryos (Rugini, 1984). This has been followed for other species (El Badaoui *et al.*, 1996; Terrer and Tomas, 2001). OM medium is rich in Ca, Mg, S, P, B, Cu and Zn, with an altered Ca/N ratio (1:11), and contains glutamine as a reduced nitrogen source. The Ca/N ratio is an important factor in olive micropropagation, as it can affect maintenance of healthy shoots (Fiorino and Leva, 1986). The requirement for boron appears to be genotype-dependent (Leva *et al.*, 1992; Dimassi, 1999). The organic composition of OM medium has been improved: (i) mannitol is a better carbon source than sucrose (Leva *et al.*, 1994); (ii) a mixture of 6-(γ,γ-dimethylallylamino)-purine (2-isopentenyladenine) (2iP), thidiazuron (TDZ) and zeatin replace the high concentration of zeatin alone; and (iii) 57.7–115.5 μM gibberellic acid (GA₃). The cultures are maintained at 23°C and 40 μmol/m²/s with an 8 h photoperiod.

Shoots are rooted on medium supplemented with either indolebutyric acid (IBA) or naphthaleneacetic acid (NAA). Shoot cultures for rooting are incubated in darkness for 5–7 days, prior to transfer to light conditions. Putrescine is involved in root induction by increasing the activity of total peroxidases at the base of explants, and promotes rapid root growth and increased rooting frequency (Rugini *et al.*, 1997). This has been attributed to the low endogenous level of polyamines in olive shoots compared to other species (Rugini, 1992). Basal treatments of explants with H_2O_2 promote early and increased rooting similar to putrescine (Rugini *et al.*, 1997). Improved production of difficult-to-root cultivars can be achieved by basal etiolation of shoots during rooting (Rugini *et al.*, 1993). *In vitro* rooting has been effective for production of plants of 'Nocellara Etnea', for which rooting of cuttings is difficult (Briccoli Bati *et al.*, 1999).

Micropropagated olive plantlets have thin cuticle, low stomatal density and a single palisade layer (Cozza *et al.*, 1997). Acclimatization under high relative humidity with continuous light is essential to obtain plants with good lateral branches, ready for transplanting. In field trials, micropropagated plants begin to flower at the same time as plants propagated by cuttings (Briccoli Bati *et al.*, 1999; Rugini *et al.*, 2000). Precocious flowering has been observed in pots, depending on the cultivar, but particularly involving polyploid (4n) genotypes (E. Rugini, personal communication). Genetic fidelity of micropropagated plants has been demonstrated. RAPD analysis (Garcia-Fèrriz *et al.*, 2000; Leva *et al.*, 2000) and agronomic and morphological observations (Briccoli Bati *et al.*, 2000; Garcia-Fèrriz *et al.*, 2000; Leva *et al.*, 2000; Rugini *et al.*, 2000) have not revealed any variation in micropropagated 'Moraiolo', 'Canino', 'Nocellara', 'Carolea', 'Maurino', 'Frantoio', 'Leccino', 'Dolce Agogia', 'Empeltre', 'Arbequina e Picual' and some north African cultivars (Table 15.1.2).

4. Elimination of Viruses

More than 70% of cultivated olive trees seem to be infected with latent viruses. At present,

about 20 viruses have been isolated from olive (Martelli *et al.*, 1995; Martelli and Prota, 1997; Martelli, 1998, 1999). There is a correlation between symptoms, i.e. twisted and narrow leaf lamina and poor growth, and the presence of strawberry latent ringspot virus (SLRV) (Marte *et al.*, 1985; Pasquini *et al.*, 2002). The production of virus-indexed olives using shoot apex culture with thermotherapy has been initiated with promising results (E. Rugini, personal communication).

5. Somatic Cell Genetics

5.1. Regeneration

5.1.1. Somatic embryogenesis

Embryogenic cultures have been induced from immature zygotic embryos (60–75 days after flowering) of various cultivars ('Dolce Agogia', 'Leccino', 'Frantoio', 'Picholine', 'Frangivento' and 'Moraiolo') (Rugini, 1988; Leva *et al.*, 1995). Somatic embryos develop directly from explants without callus formation, and high benzyladenine (BA) concentrations (2.5 μ M) inhibit somatic embryogenesis. The morphogenic potential of these explants can be sustained for at least 2 months if fruits are harvested and stored at 12–15°C (Rugini, 1995). Embryogenic cultures have also been induced from cotyledons of 126-day-old zygotic embryos of 'Chalkidikis' (Pritsa and Voyatzis, 1999), from cotyledon segments from mature zygotic embryos of wild olive (*O. europaea* var. *sylvestris*), from the radicle of mature embryos (Rugini and Tarini, 1986; Mitrakos *et al.*, 1992; Rugini *et al.*, 1995; Shibli *et al.*, 2001) and from mature zygotic embryos (Orinos and Mitrakos, 1991; Mitrakos *et al.*, 1992). There has been no standard medium for induction of these embryogenic cultures. Moreover, the light requirements for induction, maintenance and development have not been addressed, although Rugini (1988) and Shibli *et al.* (2001) attempted to define light conditions during different stages of somatic embryogenesis.

Somatic embryogenesis from tissues of elite olive has been reported for two culti-

Table 15.1.2. Micropropagation of mature tissues of cultivars by: axillary bud stimulation, shoot organogenesis and somatic embryogenesis.

Cultivar ^a	Medium (hormones)	Medium (hormones)	Result	References
Dolce	$\frac{1}{2}$ MS (zeatin + IBA + GA ₃)	$\frac{1}{2}$ Knop/Heller (NAA)	Rooting	Rugini and Fontanazza, 1981
Agogia	OM (zeatin + 2iP)	BN (NAA or IBA); (NAA + putrescine)	Rooting	Rugini, 1984, 1992
Frantoio	OM (zeatin or 2iP)	Various (NAA or IBA)	Rooting	Rugini, 1984
Moraiolo	mMS (zeatin or TIBA)	mMS (IBA – dip method)	Rooting	Bartolini <i>et al.</i> , 1990
Maurino	mOM (zeatin)	mOM (NAA)	Rooting	Rama and Pontikis, 1990
Kalamon	$\frac{1}{2}$ OM (zeatin)	$\frac{1}{2}$ OM (NAA or IBA + putrescine)	Rooting	Briccoli Bati <i>et al.</i> , 1994
Carolea	WPM (BAP + IBA + GA ₃)	WPM (IBA)	Rooting	Dimassi-Theriou, 1994
Kalamon	mOM (BAP)	Not reported	Rooting	Seyhan and Özzambak, 1994
Memecik, Domat	mOM (zeatin)	mOM (NAA)	Rooting	Briccoli Bati and Lombardo, 1995
Nocellara Etnea	OM (zeatin or 2iP)	Various (NAA + putrescine)	Rooting	Rugini <i>et al.</i> , 1996
Mixoploid Leccino and Frantoio	DKW (BAP + IBA)	$\frac{1}{2}$ DKW (IBA)	Rooting	Revilla <i>et al.</i> , 1996
Arbequina	mOM (zeatin or 2iP)	$\frac{1}{2}$ MS (NAA)	Rooting	Mencuccini, 1998
FS-17	OM (zeatin)	OM (IBA or NAA)	Rooting	Otero and Docampo, 1998
Arbequina	mMS (zeatin + kinetin or BAP)	$\frac{1}{2}$ mMS (NAA)	Rooting	Chaari-Rkhis <i>et al.</i> , 1999
Tunisian cultivars	Initial OM and OM (zeatin)	BN (IBA or NAA)	Rooting	Martino <i>et al.</i> , 1999
Moraiolo ^a	OM (BAP + TDZ)	Compost (IBA + IAA)	Rooting	Garcia-Fèrriz <i>et al.</i> , 2000
Empeltre, Picual	mOM (zeatin)	mOM (IBA – dip method)	Rooting	Chaari-Rkhis <i>et al.</i> , 2000
Meski	OM (zeatin + TDZ + BAP)	Various (NAA or IBA + putrescine)	Rooting	Zuccherelli and Zuccherelli, 2001
Various cv. ^b	OM (zeatin)	MS or mOM (TDZ or 2iP + BAP)	Shoot organogenesis and plants	Mencuccini and Rugini, 1993
Dolce	OM (zeatin)	$\frac{1}{2}$ MS (TDZ + NAA mOM (2iP) then to BAP + IBA + cefotaxime)	Somatic embryogenesis and plants	Rugini and Caricato, 1995
Agogia, Canino, Moraiolo, Halkidikis				
Canino, Moraiolo				

^a Starting material was generally nodal explants, except for Moraiolo by Martino *et al.* (1999), who started with shoot tips.

^b Arbequina, Barnea, Bella di Cerignola, Canino, Capolga, Carolea VP, Chemlali, Chétoui, Coratina, Correggiolo, Diana (R), Frantoio, Galiega, Hojiblanca, Koronenchi, Moraiolo, Nocellara del Belice, Nostrana, Ogliarola, Apollo (R) (sel. Frantoio), Arbosana, Cornicabra, Cordelli, Montefeltro II, Nebbia, Neshchi, Paravento, Pendolino, Piqua, Raggia II, S. Agostino, S. Caterina, Sury A, Sury B, Termite di Bitetto, Urano (R), Uovo di Piccione, Zeus (R) (sel. Pendolino).
OM, olive medium (Rugini, 1984); mOM, OMc, modified OM (Rugini, 1984); DKW, Driver and Kuniyuki (1984); Knop/Heller, Heller (1953); WPM, from McCown and Lloyd (1981); BN, Bourgin and Nitsch (1967).

vars, 'Canino' and 'Moraiolo' (Rugini and Caricato, 1995).

Induction. Embryogenic cultures have been induced from organogenic leaf petioles of micropropagated plantlets (see section 5.1.2) (Rugini and Caricato, 1995). Induction conditions consist of semi-solid modified OM (OMc) medium supplemented with growth regulators (1 g/l casein hydrolysate and 3% sucrose) in darkness.

Maintenance. Embryogenic cultures consisting of proembryonal masses (PEMs) can be maintained on filter paper soaked with liquid OMc medium and, following subculture on to semi-solid, hormone-free OMc medium with 0.1% (w/v) activated charcoal, cycles of secondary somatic embryogenesis can be maintained for several years with monthly subculture. Secondary somatic embryos originate from the epidermis or rarely from the first subepidermal layer of embryos (Lambardi *et al.*, 1999a). A limited number of cells of the primary explant is apparently involved in the formation of somatic embryos (Benelli *et al.*, 2001a).

Maturation and germination. There have been no systematic studies of somatic embryo maturation; however, abscisic acid (ABA) has been used to synchronize embryo formation. Somatic embryo germination is achieved following their transfer to liquid OMc medium, where they are maintained at 80 rpm. Conversion rate declines with time, although root elongation occurs readily in liquid medium containing 1.36 μM zeatin. Various attempts to increase the conversion rate, including chilling and growth regulator inhibitors, have been ineffective (E. Rugini, personal communication).

5.1.2. Organogenesis

Organogenesis has been induced from zygotic embryos and from mature phase explants of olive cultivars. The frequency of organogenesis from both types of materials is low; however, organogenesis is an essential first step for somatic embryogenesis since embryogenic

cultures are derived from leaflets of adventitious buds that develop from leaf petioles of micropropagated plantlets.

Induction. Bao *et al.* (1980) induced organogenic cultures from hypocotyl sections. Later, organogenesis was reported from cotyledon fragments proximal to the embryo axes of mature embryos on mOM medium with a high auxin (IBA)/cytokinin (2iP or zeatin) ratio (Rugini, 1986; Canas and Benbadis, 1988). Shoot development was stimulated when the callus was transferred to OM medium containing 19.7 μM 2iP. Rooting of individual shoots occurred on OM medium containing either 4.9 μM IBA or 5.37 μM NAA.

Regeneration from mature phase explants of important cultivars (Table 15.1.2) has been described by Mencuccini and Rugini (1993) on MS semi-solid medium and on modified OM medium in darkness. The highest regeneration was achieved directly from the proximal part of petioles after 2–3 weeks on medium containing 5–40 μM TDZ or with 10 μM 2iP plus 2.2 μM BA with or without a low auxin concentration (not more than 2.5 μM). The regeneration potential is higher from petioles collected from apical nodes than from basal ones. For further development, the adventitious shoots can be transferred to semi solid half-strength MS medium supplemented with 4.5 μM zeatin before transfer to OM medium for shoot proliferation. Several regenerated shoots have been rooted and the plantlets have been hardened in the greenhouse. No apparent differences regarding morphological aspects were observed among them or with plants produced by axillary bud stimulation.

5.1.3. Haploid recovery

Homozygous plants would be of great interest for isolation of mutants and recessive traits. Classic methods to obtain homozygosity by self-pollination are hampered by self-sterility and long juvenile period. Despite efforts to recover haploids of olive, there have been no reported successes (Mulè *et al.*, 1992; Perri *et al.*, 1994a).

5.1.4. Polyploids

Triploid olive plants have been produced by fertilizing tetraploid plants with normal olive pollen. Tetraploid plants have been produced from mixoploid (2n and 4n cells) shoots from irradiated 'Leccino' and 'Frantoio' plantlets. During *in vitro* proliferation of irradiated shoots, tetraploid and diploid shoots can be recovered (Rugini *et al.*, 1996). Triploid plants have been isolated from the largest fruits harvested from mixoploid plants or from 4n plants, both of which have been pollinated with (haploid) pollen.

5.1.5. Protoplast isolation and culture

Although viable protoplasts have been isolated from hypocotyls, cotyledons and leaves of micropropagated shoots, and in some cases microcalluses have been obtained, regeneration has not been reported (Rugini, 1986; Canas *et al.*, 1987; Mencuccini, 1991; Perri *et al.*, 1994b).

5.2. Genetic manipulation

5.2.1. Mutation induction

Roselli and Donini (1982) irradiated rooted cuttings of 'Ascolana Tenera', and obtained dwarf plants. Mixoploid mutants have been obtained following irradiation of 'Frantoio' and 'Leccino' plantlets (Pannelli *et al.*, 1990), resulting in the selection of dwarf 'Leccino', which is self-sterile and late blooming (Pannelli *et al.*, 1990) and seems to be promising as a rootstock.

5.2.2. Genetic transformation

Genetic transformation is potentially the most powerful technique for accelerating the development of superior olive cultivars.

Breeding objectives. Genetic transformation of olive has focused on problems that affect cultivation, productivity and processing. The targeted traits include: (i) self-fertility; (ii) oil content and composition; (iii) parthenocarpy; (iv) abiotic stress tolerance;

(v) plant habit; (vi) fruit ripening; and (vii) resistance to pathogens and pests. Several genes of different origin that mediate these characters have been identified; however, development of efficient regeneration methods from mature tissue of elite selections remains the limiting factor for using this technology for a range of cultivars.

Juvenility. Several efforts have been devoted to accelerate the flowering time, and some genes controlling flower initiation in *Arabidopsis* have been identified (Yanotsky, 1995; Simpson *et al.*, 1999). Two of them, LEAFY (LFY) and APETALA1 (AP1), have been used to transform *Citrus* (Pena *et al.*, 2001), resulting in flower initiation in 1-year-old plantlets.

Oil content and composition. Currently, two strategies are used to modify oil composition and content: (i) alteration of the major fatty acid level by suppressing or expressing a specific key enzyme in lipid biosynthesis; and (ii) creation of an unusual fatty acid. By antisense suppression or co-suppression of oleate desaturase, it was possible to increase oleic acid (C18:1) by more than threefold (from 24% to 80%) in the oil of transgenic soybean. The same strategy was adopted to increase stearic acid (C18:0) by up to 30% in canola and soybean oils. Unusual fatty acids can be produced in one plant by transferring a gene encoding the specific biosynthetic enzyme, e.g. canola does not produce laurate (C12:0), while a new transgenic genotype contains laurate. The oil content could thereby be increased or the composition could be modified.

Parthenocarpy. Several olive cultivars, e.g. 'Giarrappa', are naturally parthenocarpic, although fruits usually do not develop normally and are undersized. Genes inducing parthenocarpy would permit the development of parthenocarpic fruits with regular size, and would overcome the problem related to self-sterility among olive cultivars. Self-sterility is under the control of the placental-ovule-specific *def19* gene regulator sequence expressed during early flower development (Rotino *et al.*, 1997).

Abiotic stress tolerance. Cold-hardiness is a very important objective for olive improvement (Bartolozzi and Fontanazza, 1999). Transformation could involve the anti-freeze protein (Hightower *et al.*, 1991), the over-expression of the superoxide dismutase (SOD) gene, which permits the repair of frost-damaged cells (McKersie *et al.*, 1993; Van Camp *et al.*, 1994), and the CBF1 gene increases cold tolerance by inducing genes related to cold-hardening (Jaglo-Ottosen *et al.*, 1993).

Architecture. Reduced plant size and altered tree architecture would increase olive planting density and make the trees more suitable for mechanical pruning and harvesting. *Agrobacterium rhizogenes* TL-DNA in kiwifruit, cherry (Gutierrez-Pesce *et al.*, 1998; Rugini *et al.*, 1999) and peach (Hammerschlag and Smigocki, 1998) and phytochrome genes (*phyA* and/or *phyB*) in sense and antisense reading frames can control plant development to result in plants with high agronomic value and suitable for intensive cultivation systems (Baraldi *et al.*, 1992; Muleo and Thomas, 1997).

Fruit ripening. Most olive cultivars show problems in fruit ripening, i.e. non-synchronized maturation, high water content, immature fruit drop and low fruit pulp firmness. The introduction of genes in the antisense reading frame that block ethylene synthesis (Oeller *et al.*, 1991) or reduce polygalacturonase activity could be useful (Smith *et al.*, 1988).

Resistance to pathogens and pests. Wide-spectrum resistance to diseases and pests is difficult to obtain by traditional breeding methods. Anti-fungal genes or a pool of these genes may be effective for improving resistance to fungal diseases, particularly *Verticillium* wilt and *Spilotea oleagina*. *Osmotin* gene, stilbene synthase (Hain *et al.*, 1993), protein-inactivating ribosomes (Longmann *et al.*, 1992), glucose oxidase (Wu *et al.*, 1995) or genes for hydrolytic enzymes, i.e. chitinase and glucanase (Broglie *et al.*, 1991; Yoshikawa *et al.*, 1993), and proteins inhibiting polygalacturonase (PGIPs) should

improve the olive defence system. Increased resistance of other species to bacterial diseases has been obtained by introducing genes for bactericidal polypeptides, i.e. cecropin (Huang *et al.*, 1997) and its synthetic analog MB39 (Mills *et al.*, 1994), thionin (Carmona *et al.*, 1993), *attacin E* (Norelli *et al.*, 1994) and human lysozyme (Nakaijima *et al.*, 1997). The isolation of homologous genes conferring resistance to fruit fly (*Bactrocera oleae*) from olive cultivars with low susceptibility to this insect is a potential way to achieve resistance to this pest.

Protocol. Reports of transformation of olive have involved zygotic and sporophytic material and have utilized either *Agrobacterium* or a biolistic approach. The first studies produced chimeric plants with transformed roots using wild type *A. rhizogenes* (Rugini, 1986, 1992). Subsequently, immature zygotic embryos were used to produce transformed somatic embryos, with limited success (Rugini and Fedeli, 1990; Mencuccini *et al.*, 1997). Transgenic callus was produced from leaf petioles of *in vitro* shoots (Mencuccini *et al.*, 1999). Somatic embryos from which secondary embryos develop (Rugini and Caricato, 1995) are the most suitable tissues for transformation (Lambardi *et al.*, 1999b; Rugini *et al.*, 1999).

Most transformation experiments involving olive (Table 15.1.3) that have utilized *Agrobacterium* were carried out with *rol* genes of *A. rhizogenes* T-DNA cloned in *A. tumefaciens* LBA4404 containing the plasmid pBIN19 (Bevan, 1984). Cefotaxime (200 mg/l) has been used to eliminate *A. tumefaciens* following co-culture (Rugini and Caricato, 1995). Kanamycin (50–100 µg/ml) has been used to select transformed cultures. Transformation efficiency can be improved by co-culture in a shaker with carborundum powder (Rugini *et al.*, 1991) followed by incubation with acetosyringone. Lambardi *et al.* (1999b) used a biolistic approach to transform 'Canino' somatic embryos. The highest levels of transient glucuronidase (GUS) gene expression were observed when somatic embryos (> 5 mm length) were bombarded with the pZ085 and pCGU80 plasmids at 580 kPa.

Table 15.1.3. Genetic transformation in olive.

Goals	Cultivar	Explant	System (gene)	Promoter	Plasmid	Regeneration	Authors
Chimeric plants	Dolce	Shoots	<i>A. rhizogenes</i> (T-DNA)	Natural	pBIN19	Roots	Rugini, 1986, 1992
Reporter gene	Agogia	Somatic embryos	Particle gun (<i>gus</i>)	35S and ubiquitin	pZO85 pCGUS	Transient expression	Lambardi <i>et al.</i> , 1999a
Growth habit modifications	Moraiolo	Zygotic embryos	<i>A. tumefaciens</i> LBA4404 (<i>rol ABC</i>)	Natural	pBIN19	Callus	Rugini and Fedeli, 1990
	Canino	Somatic embryos of cv.	<i>A. tumefaciens</i> LBA4404 (<i>rol ABC</i>)	Natural	pBIN19	Somatic embryos and plants	Rugini, 1995; Rugini <i>et al.</i> , 1999
	Dolce Agogia	Petioles	<i>A. tumefaciens</i> LBA4404 (<i>rol ABC</i>)	Natural	pBIN19	Callus and roots	Mencuccini <i>et al.</i> , 1999
Disease resistance	Canino	Somatic embryos of cv.	<i>A. tumefaciens</i> LBA4404 (<i>Osmotin</i>)	35S	pKYLX71	Plants	Rugini <i>et al.</i> , 1999
Disease resistance	Canino	Somatic embryos of cv.	<i>A. tumefaciens</i> LBA4404 (<i>Osmotin</i> + <i>Chitinase</i> + <i>PR1</i>)	35S	pKYLX71	Plants	E. Rugini <i>et al.</i> (unpublished)

The neomycin phosphotransferase II (*nptII*) gene, encoding resistance to the antibiotic kanamycin, has been used for olive; selection of transformed cells is usually carried out with 50–100 mg/l kanamycin. *In vitro* olive shoot growth is arrested at 30–40 mg/l kanamycin, while 20–25 mg/l kanamycin inhibits greening of somatic embryos when they are transferred from dark to light conditions. Selection of transformed somatic embryos still attached to PEMs on semi solid medium requires > 100 mg/l kanamycin.

Accomplishments. Olive has been transformed with: (i) the entire T-DNA of *A. rhizogenes* to improve rooting efficiency; (ii) the *rolABC* genes of *A. rhizogenes*, to modify plant morphology; (iii) the *osmotin* gene, to provide defence against fungal pathogens; and (iv) *gus* and *nptII* markers, to select or to evaluate gene promoters. Transformed plants have been regenerated and they are under field evaluation.

Increased rooting efficiency. Rooting was induced by inoculating the basal part of microshoots with 'wild type' *A. rhizogenes* (Rugini and Fedeli, 1990; Rugini, 1992; Rugini and Mariotti, 1992). Putrescine

increased rooting and basal callus formation (Rugini, 1992), although only a low percentage of plants (< 20%) had agropine-positive roots. Regenerated plants are under observation for altered growth characteristics.

Growth habit modification. Immature zygotic embryos of 'Moraiolo' 75 days after flowering and embryogenic 'Canino' cultures were transformed with *A. tumefaciens* strain LBA 4404 containing the *rolABC* genes of *A. rhizogenes* and the *nptII* gene in a pBin19 construct. Putative transformed somatic embryos and morphogenic callus were selected with kanamycin (Rugini and Fedeli, 1990; Rugini and Caricato, 1995; Rugini *et al.*, 1999). Following selection on semi-solid medium with kanamycin, somatic embryo development was initiated. Mature somatic embryos were transferred to liquid medium containing zeatin under light conditions to stimulate germination. Plantlets from germinated somatic embryos and rooted microcuttings were transplanted to potting mixture and hardened before transfer to the greenhouse.

Disease resistance. Embryogenic cultures were infected with *A. tumefaciens* strain

LBA4404 containing the *osmotin* gene from tobacco in pKYLX71 with the cauliflower mosaic virus (CaMV) 35S promoter (Rugini *et al.*, 1999). In the field, olive plants expressing *osmotin* have shown reduced growth (Rugini *et al.*, 2000; D'Angeli *et al.*, 2001), which has also been reported for apple plants transformed with the endochitinase gene (Bolar *et al.*, 2000). Experiments are in progress with three genes from tobacco (*osmotin* + *chitinase* + *PR1*) in one construct under the 35S promoter. Transformed somatic embryos have been selected and plants have been established in the greenhouse (Rugini *et al.*, 2000).

5.3. Cryopreservation

Successful encapsulation of shoot apices of olive has been reported (Micheli *et al.*, 1998). This technology may be suitable for short- and medium-term storage of olive germplasm.

Cryogenic methods have been applied to olive for long-term conservation of germplasm. Martinez *et al.* (1999) observed 30% survival of 'Arbequina' shoot tips following their desiccation to 30% moisture content and direct immersion into liquid nitrogen. Lambardi *et al.* (2000) utilized vitrification and one-step freezing in liquid nitrogen for shoot tips excised from *in vitro* 'Frantoio' shoot cultures. Following rewarming at 40°C and plating on regrowth medium, 15% survival was achieved, but only from shoot tips which had been obtained from apical buds. The surviving shoot tips remained green and started to grow 4 weeks after plating.

Embryogenic 'Canino' cultures, consisting of PEMs and somatic embryos at various stages of development, can be cryopreserved by vitrification (Lambardi *et al.*, 2000). After incubation in vitrification solution, a high percentage (38%) of the cryopreserved cultures can survive. Cryopreserved cultures show enhanced proliferation and morphogenic potential. The encapsulation-dehydration procedure has been ineffective for cryopreservation of 'Frantoio' (Benelli *et al.*, 2001b) and 'Arbequina' (Martinez *et al.*, 1999).

6. Conclusions

Several olive cultivars have been successfully micropropagated, and the technique is used commercially. Significant progress has also been made in the refinement of somatic embryogenesis from mature phase tissues of elite cultivars. Promising results with respect to medium- and long-term conservation of olive by slow growth storage and cryopreservation will be useful for the establishment of an olive *in vitro* repository, which could safeguard olive biodiversity. For olive transformation, the critical areas for the future include: (i) identification and evaluation of genes and specific promoters for useful traits; and (ii) development of efficient regeneration protocols for elite cultivars.

Many potentially useful genes have already been isolated from several species, and could be introduced into olive separately or as multiples. Transformation experiments with multiple genes are currently under way. The transformation selection procedure should be improved to increase public acceptance, possibly by replacing traditional selection markers (*nptII*) with other markers (*gfp*, *lecl*, *ipt*, *pmi*, *xyla*), although the percentage of escapes is quite high (>40%) in fruit crops (May *et al.*, 1995; Pena *et al.*, 1995, 1997; Mourgues *et al.*, 1998; Perl and Eshdat, 1998). Endo *et al.* (2001) suggested that the *ipt* gene from *A. tumefaciens* might be a suitable selectable gene. A multi-autotransformation (MAT) vector, which combines the use of genes that stimulate growth and morphogenesis for positive selection of transformed cells with an excision mechanism to remove the markers and allow recovery of plants with normal phenotypes, could be very useful. The vector, which includes *ipt* and the maize transposable element *Ac* for removing the *ipt* gene, seems to be promising (Ebinuma *et al.*, 1997). Other approaches might involve *xyla* (xylose isomerase) (Haldrup *et al.*, 1998) and *pmi* (phosphomannose isomerase) genes, both of which confer the capacity to use non-metabolizable substrates. Finally, for highly regenerative cultures, it may be possible to eliminate the marker gene and select on the basis of physiological or morphological parameters, e.g. the response to toxins,

culture filtrate, salt/drought resistance, etc. (Graham *et al.*, 1996).

Molecular markers are likely to be used for evaluating the level and distribution of

olive germplasm variability and for identifying markers linked to important agronomic and quality traits to accelerate breeding programmes.

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16

Oxalidaceae

The *Oxalidaceae* is a heterogeneous family, comprised of approx. seven genera and 1000 species. It is a family consisting primarily of tropical shrubs and herbaceous species, but with a few small tree species. Only a few species within the family are important, and these include the Andean tuber crop oca (*Oxalis tuberosa* Mol.), ornamental *Oxalis* shrubs and herbaceous species, e.g. *O. hedysarioides* HBK, ornamental *Biophytum* species, e.g. *B. sensitivum* (L.) DC, the bilimbi (*Averrhoa bilimbi* L.) and carambola or star fruit (*Averrhoa carambola* L.) (Watson and Dallwitz, 1992 onwards). Hutchinson (1959) proposed that the *Averrhoa* species should be placed in a sepa-

rate family, the *Averrhoaceae*; however, this has not been accepted. A common characteristic of many species within the family is the presence of high levels of oxalic acid in tissues throughout the plant. This confers the sourness that is typical of the carambola fruit. The bilimbi and carambola are closely related, and both species originated in South-East Asia, most probably in Malaysia (Popenoe, 1924; Burkill, 1966) and Indonesia (Purseglove, 1968), respectively. The bilimbi or cucumber tree is grown as a commercial crop on a limited scale in South-East Asia. Unlike the carambola, the sourness of bilimbi is associated with high levels of citric acid.

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16.1 *Averrhoa carambola* Carambola

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1. Introduction

1.1. Botany and history

The carambola is a small understorey tree of the South-east Asian rainforest attaining a maximum of 15 m height. The compound leaves are imparipinnate with entire, ovate leaflets (three- to six-jugate) (Samson, 1992). Flowers usually occur in panicles in axils, are rarely cauliflorous and are heterodistylous (Ghose and Dhua, 1990; Samson, 1992). Carambola fruit is a berry. The juvenile period is 5–6 years, although vegetatively propagated trees can flower in 2–3 years. Flowering is almost continuous, with normally two harvest periods annually in many growing areas. The trees are considered to be heavy bearers. The fruit is strongly acidic, which is attributable to high levels of calcium oxalate. Among various cultivars, the taste ranges from sweet to slightly acidic to very sour.

1.2. Importance

The carambola fruit is consumed whole or sliced as a garnish in salads and iced drinks. From its original home in South-east Asia, the carambola has spread to most lowland tropical regions of the world. Production

data for this crop are unavailable from FAO-STAT (2001); however, it is an important crop in Malaysia, Indonesia, the Philippines, Vietnam and Thailand. There is also significant production of carambola in China, India, the USA, Australia and South and Central America. Limited amounts of fresh fruit are exported to Japan and the European Union (EU).

1.3. Breeding and genetics

According to Knight (1965, 1982), carambola flowers with long styles are self-fertile, whereas flowers with short styles require pollination with pollen from flowers with long styles, although self-incompatibility is not absolute. As a result, for best yields, the two types of trees are often planted together. Partial self-incompatibility is considered a hindrance for inbreeding cultivars with short styles. Rahman (1996) has indicated that selection for self-compatibility is a priority in Malaysia.

There has been only a limited effort to develop cultivars through conventional breeding, primarily in the USA and Malaysia. As a result, there is little genetic information for this species ($2n = 2x = 22, 24$). Modern cultivars have been selected from within populations of openly polli-

nated seedlings or from 'dooryard' seedling trees. Flowering and fruit set differ significantly between acidic and sweet carambola selections (Nand, 1970, 1971). There are twice as many flower panicles among sweet cultivars in comparison with sour or acidic cultivars. Moreover, fruit set is greater in sweet cultivars than in acidic or sour cultivars.

1.3.1. Rootstocks

Major breeding objectives. There are no major breeding objectives that could stimulate the development of improved rootstock selections. However, Knight (1982) demonstrated that seedlings from openly pollinated cultivars that are able to tolerate calcareous soils also appear to tolerate these soil conditions better than the progeny of cultivars selected for their performance on different soil types. This should facilitate screening for certain types of soil stress tolerance in seedling populations of known parentage.

1.3.2. Scions

Major breeding objectives.

Oxalic acid. The most important breeding goal for developing improved carambola cultivars is the reduction in the levels of oxalic acid in fruit. The oxalic acid concentration in fruit of some carambola cultivars is unacceptably high from a health perspective. The amount of oxalic acid is highly variable among seedlings of a common parent (Wilson *et al.*, 1982) and among different cultivars (Rahman and Esa, 1996). The concentration of oxalic acid in fresh carambola fruit can vary from <0.10 g/100 g fresh weight (FW) with the sweet 'Fwang Tung' to approx. 0.7 g/100 g FW of the seedling tree WA3-24-37 (Wilson *et al.*, 1982). Reduction of oxalic acid concentrations is important if the fruit is to become acceptable in developed country markets, not only as a garnish for fruit salads and drinks, but as a fruit that can be consumed in quantity. According to Wilson *et al.* (1982), the relative sweetness and sourness of carambola fruit are not inversely correlated. For example, 'Newcombe' and 'Arkin' are

both sweet cultivars; however, the concentration of oxalic acid is actually much higher in 'Newcombe' than in 'Arkin'. Therefore, it is the absolute concentration of sugars that determines sweetness, irrespective of the level of oxalic acid.

Postharvest losses. The carambola fruit is non-climacteric, and therefore ripening cannot be initiated if the fruit are picked green. Fruit are usually harvested just before colour change or at maturity, and can be stored for up to 4 weeks at 5–10°C under high relative humidity (RH). Many carambola cultivars have fruit with thin or narrow ribs, which are easily injured during picking, in transit to packing houses and when otherwise handled. Slightly damaged fruit are highly susceptible to diseases in storage. Carambola fruit under postharvest conditions is infected by *Ceratocystis*, *Colletotrichum*, *Dothoriella* and *Phomopsis* (Samson, 1992). Postharvest losses could be minimized if ripening could be controlled and if fruit shape could be altered in order to minimize bruising or damage to the fruit ribs.

Disease resistance. Although several diseases affecting carambola fruit and foliage have been reported, these are not considered to be major production problems. However, postharvest rots are extremely serious (see previous section). Fruit are very easily injured during harvesting and storage. The slightest bruise is often sufficient to provide a site for infection by a plant pathogen that will result in serious postharvest losses. Enhanced resistance to plant pathogens causing postharvest losses would be a significant advance.

Breeding accomplishments. Conventional breeding has had no impact on carambola improvement. All cultivars are seedlings from chance or open pollinations. The tree has not been domesticated; there is very little difference between trees in cultivation and trees in the wild in their native habitat. Due to its heterozygosity, there is considerable genetic diversity within the species. This has enabled the selection of several commercial cultivars that have become distributed in the

major growing areas. The superior selections generally have sweet flesh and thick ribs.

2. Somatic Cell Genetics

2.1. Regeneration

There have been several reports in which the *de novo* regeneration of carambola plantlets, from either juvenile or mature phase explants, has been described. The rationales of many of these studies, i.e. to form the basis for genetic manipulation of this plant and the quality of its fruit or for micropropagation, are not clear.

2.1.1. Organogenesis

Juvenile explants. The regeneration of carambola shoots from organogenic calluses derived from embryonic or juvenile tissues has been described in various reports, including Litz and Conover (1980), Rao *et al.* (1982), Smith *et al.* (1987), Kantharajah *et al.* (1992), Amin and Razzaque (1993), Khalekuzzaman *et al.* (1995a,b) and Islam *et al.* (1996). Different explants have been utilized, including cotyledons (Litz and Conover, 1980; Khalekuzzaman *et al.*, 1995b) and hypocotyls (Islam *et al.*, 1996). Although these regeneration pathways are not necessarily applicable to elite (mature phase) trees, they could form the basis for procedures such as somatic hybridization, which could be used for development of improved root-stock selections.

Elite explants. Regeneration of elite carambola selections is a prerequisite for applying somatic cell genetic approaches to existing cultivars. Induction of shoot organogenic callus from leaflets of compound leaves of mature phase 'Arkin' carambola was reported by Litz and Griffis (1989) and Griffis and Litz (1993). Stock plants were grafted 'Arkin' plants that were maintained in a greenhouse. Trees were hard pruned in order to stimulate new leaf flushes, and the foliage was sprayed with 200 mg/l benzyladenine (BA). Following their surface steril-

ization, 1 cm diameter circular explants were cut from the youngest, fully expanded leaflets and were placed on semi-solid Murashige and Skoog (1962) (MS) medium that was supplemented with 30 g/l sucrose, 24.6 μ M 6-(γ,γ -dimethylallylamino) purine (2iP) and 22.6 μ M 2,4-dichlorophenoxyacetic acid (2,4-D) to stimulate organogenic callus induction and proliferation. All explants were transferred to fresh medium 6 weeks after explanting and thereafter at 4-week intervals. After 14 weeks on induction medium, callus was transferred to semi-solid medium supplemented with 30 g/l sucrose and 24.6 μ M 2iP. Shoot regeneration was apparent 2 weeks after subculture from medium without 2,4-D. Limited shoot proliferation was initiated by subculture of shoots on semi-solid medium with 24.6 μ M 2iP and 2.85 μ M indoleacetic acid (IAA). Rooting of proliferated shoots was not obtained.

Although Kantharajah *et al.* (1992) published a report regarding regeneration of carambola shoots from root callus purportedly derived from mature phase plants, in fact the source(s) of the explants were roots from 3-year-old seedlings. According to Samson (1992), the juvenile period of carambola is 5–7 years. Therefore, it is not certain that this protocol is directly applicable to regeneration from roots from mature phase trees; however, this question should be addressed and confirmed. Kantharajah *et al.* (1992) treated seedlings grown in 30 gallon pots with 2 g/l systemic fungicide (Fongarid 250 WP), removed root cuttings (without the root tips), washed them in running water and surface sterilized them in 30% (v/v) commercial bleach. Following surface sterilization, the root segments were rinsed thoroughly with sterile distilled water, and cultured on semi-solid MS medium supplemented with 20 g/l sucrose, 8.9 μ M BA and 1.1 μ M naphthaleneacetic acid (NAA) for induction of organogenic cultures. Root induction was observed following transfer of individual shoots on to semi-solid MS medium supplemented with 20 g/l sucrose and 1% (w/v) activated charcoal. The shoots regenerated from callus cultures by Litz and Griffis (1989), Kantharajah *et al.* (1992) and Griffis and Litz (1993) were unable to be rooted either *in vitro*

or *ex vitro* in potting medium. However, plantlets derived from cultured roots survived transfer to potting mixture in the greenhouse (Kantharajah *et al.*, 1992).

3. Conclusions

The carambola is considered to be an under-exploited fruit crop with great potential. It is important in many countries in South-east Asia, and is increasingly being grown outside this region. The carambola fruit has great unrealized possibilities as an export crop from many tropical and subtropical producing regions to North America, the EU and Japan. In order for its potential to be realized, however, there are several plant breeding objectives that must be addressed: (i) control of fruit ripening of this non-climacteric fruit; (ii) control of fruit rib morphology in order to minimize damage; (iii) control of postharvest

losses caused by plant pathogens; and (iv) reduction in the concentration of oxalic acid in fruit. Objectives iii and iv could be addressed using currently available somatic cell genetic approaches. The targeting of oxalic acid levels and lowering these levels in carambola fruit is feasible according to the protocol of Thompson *et al.* (1994), who described the genetic transformation of canola (*Brassica napus*) with oxalate oxidase and the resulting lowering of the endogenous levels of oxalic acid. The fruit of carambola could be improved significantly using currently available biotechnologies, including genetic transformation and *in vitro* mutation induction and selection.

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17

Passifloraceae

The *Passifloraceae* of the order *Passiflorales* and tribe *Passiflorae* is comprised of > 600 species in 18 genera. Although 50 species bear edible fruit, only the two forms of *Passiflora edulis*, i.e. the purple and the yellow forms, are considered to be of value in international commerce. There are approx. 465 *Passiflora* spp. (Vanderplanck, 1996). All species are herbaceous or woody vines, usually with axillary tendrils. They are distributed in North and South America, the Caribbean region, the Galapagos Islands, Africa, Australia, the Philippines, Asia and Oceania. South America is the centre of diversity of the *Passiflora* genus with 95% of all species (Vanderplanck, 1996; Nakasone and Paull, 1998), and approx. 40 species are indigenous to Asia and the South Pacific islands.

Passiflora species have easily recognized bisexual flowers. The fruit is a capsule or berry containing numerous seeds with a pit-

ted testa surrounded by a fresh aril. Plants have tendrils to aid their climbing habit and their alternate leaves often have glandulate petioles. The presence, shape and number of nectar-yielding glands on the petiole are the main features that separate species and groups. The classification proposed for the genus by Vanderplanck (1996 and references therein) included 24 *Passiflora* subgenera. This has been modified by MacDougal and Feuillet (Missouri Botanical Garden, St Louis, Missouri, USA), who suggested that there are four subgenera. Several *Passiflora* spp. are important for their nutritional and pharmacological properties as their leaves contain alkaloids. Flowers of many species are grown as ornamentals. Substantial diversity is known to exist within and between species, which can be exploited in breeding programmes for improving flowering, productivity, resistance to pests and diseases, cold tolerance and fruit characteristics.

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17.1 *Passiflora* spp. Passionfruit

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1. Introduction

1.1. Botany and history

Passion vines are evergreen or semi-evergreen climbers, grown for their edible fruits, and several species are cultivated for their unusual and beautiful flowers. Vigorous, wood-stemmed and tendril climbers, nearly all *Passiflora* spp. have large, complex, very showy flowers from spring to autumn. The juvenile period is approx. 90 days. Leaves are evergreen, usually alternate, rounded-oblong or oval to lanceolate. Flowers are deep scarlet, white, pink, red, purple or pale violet associated with a red, pink, amethyst or white banded (or not) corona of filaments. Table 17.1.1 includes a list of *Passiflora* spp. that are either cultivated or have commercial potential.

Passionflowers are insect-pollinated (*Xylocopa* spp.). *Passiflora edulis* f. *flavicarpa* is normally self-incompatible (Knight and Winters, 1962; Bruckner *et al.*, 1995), and there is evidence that this is controlled by two loci, the S gene and a gametophytic gene, which acts in association with the sporophytic gene (Rego *et al.*, 1999, 2000). Flowers are perfect, and are classified in three types in *P. edulis* according to the curvature of the style: totally curved (TC), partially curved (PC) and with a standing

style (SC), of which TC is the most common. Carpenter bees are efficient pollinators of TC and PC flowers. SC flowers do not set fruit, although they produce fertile pollen grains (Ruggiero *et al.*, 1978).

Heliconid butterflies lay their eggs on passion vines, and their larvae devour the leaves. As defensive strategies, passion vines alter leaf shape or accumulate cyanide in their leaves, or change the shape and colour of the nectar glands to resemble eggs. Some species enlist the help of ants to protect them from caterpillars, and the nectar glands are either underneath the leaves or on the leaf stalk to reward ants (Gilbert, 1983).

Passiflora can be propagated quite readily by seeds, cuttings and grafting on to seedling rootstock. Plants of the yellow form are almost exclusively seed-propagated, and seeds germinate in approx. 2–3 weeks.

Passionfruits are adapted to a wide range of climates (0–35° latitude) and altitudes (0–3200 m) with high and well-distributed annual rainfall. Edible species are grown in warm tropical and cool subtropical regions and tolerate various soil types. The purple passionfruit crops best at higher altitudes (1200–2000 m) and can be grown in the subtropics, while the yellow form and its hybrids prefer tropical lowlands. The plants are very sensitive to wind damage and frost, although *P. incarnata* and *P. caerulea* can

Table 17.1.1. Commercial *Passiflora* species, their origin and uses (<http://www.ciat.cgiar.org/ipgri/>).

Species	Status	Origin	Uses
<i>P. alata</i>	Cultivated	Brazil, Venezuela, Amazonian region	Aril eaten fresh
<i>P. caerulea</i>	Rootstock	S. Brazil, N. Argentina, Paraguay,	Edible fruit
	Ornamental	Uruguay	
<i>P. capsularis</i>	Wild	C. America, Antilles, Colombia,	Edible fruit
		C. Brazil to Paraguay, Argentina	
<i>P. cincinnata</i>	Ornamental	Tropical and subtropical regions of	Edible fruit, ornamental, tea
		S. Latin America	or 'guarapo', medicinal
<i>P. coccinea</i>	Ornamental	Venezuela, Guyana, Amazonian	Aril eaten fresh, juice,
		Peru, Bolivia, Brazil	ornamental
<i>P. cumbalensis</i>	Wild	Andean Colombia to N. Peru	Edible fruit
	Cultivated	highlands around Bogota	
<i>P. cuneata</i>	Wild	Mountains of Venezuela, and	Fruit eaten fresh, medicinal
		Colombia (700–3800 m)	
<i>P. cyanea</i>	Collected	Trinidad, N. Venezuela	Fruit eaten fresh
<i>P. edulis</i>	Cultivars	S. Brazil	Aril eaten fresh, medicinal
<i>P. edulis</i>	Cultivars	S. Brazil	Industrialized juice, sedative
<i>P. fieldiana</i>	Wild	Venezuela: 'Aragua' state (endemic)	Edible fruit, eaten fresh
<i>P. filamentosa</i>	Cultivated	C. Brazil	Aril eaten fresh
<i>P. foetida</i>	Collected	Tropical America	Aril eaten fresh, juice,
			cover plant
<i>P. gabrielliana</i>	Wild	French Guyana	Aril edible
<i>P. glandulosa</i>	Wild	Guyana	Aril eaten fresh
<i>P. incarnata</i>	Cultivated	S.E. United States	Aril eaten fresh, sedative
<i>P. laurifolia</i>	Cultivated	N. Latin America, Antilles	Aril eaten fresh
<i>P. ligularis</i>	Cultivated	Andean Venezuela to Peru and	Edible fruit,
		Bolivia, C. America	aril eaten fresh
<i>P. maliformis</i>	Cultivated	N. Latin America	Aril eaten fresh, beverages
			and desserts
<i>P. mandoni</i>	Wild	Bolivia	Edible fruit
<i>P. manicata</i>	Genetic	Andean Venezuela to Peru	Possible rootstock
	resource		
<i>P. membranacea</i>	Wild	Mexico, Costa Rica	
<i>P. mixta</i>	Collected	Andean Venezuela to Bolivia	Aril eaten fresh
<i>P. mooreana</i>	Wild	Argentina, Paraguay, Bolivia	
<i>P. nigradenia</i>	Genetic	Amazon, Brazil, Bolivia	Edible fruit
	resource		
<i>P. nitida</i>	Cultivated	Antilles and N. Latin America	Aril eaten fresh
		(Guyana to Peru)	
<i>P. organensis</i>	Wild	S.E. Brazil	Pastry
<i>P. peduncularis</i>	Wild	Peru	Edible fruits
<i>P. pergrandis</i>	Collected	S. Amazonian Ecuador	Edible fruits
<i>P. pinnatistipula</i>	Cultivated	Andean Peru, Bolivia, Chile	Aril eaten fresh
<i>P. platyloba</i>	Genetic	Central America	Soft drinks
	resource		
<i>P. popenovii</i>	Cultivated	S. Colombia and Ecuador	Aril eaten fresh
<i>P. quadrangularis</i>	Cultivated	Tropical Americas	Edible fruit, juice
<i>P. retipetala</i>	Wild		Fresh fruit and juice, tea or
			'guarapo', medicinal
<i>P. riparia</i>	Collected	Amazon, Brazil	Edible fruit, aril eaten fresh
<i>P. rubra</i>	Wild	Antilles, Central America, Brazil, Peru	Fruit eaten fresh
<i>P. seemanni</i>	Collected	Colombia, Central America, Panama,	Edible fruit, aril eaten fresh,
		Mexico, Nicaragua	juice
<i>P. serratifolia</i>	Wild	Central America	Edible fruit, aril eaten fresh
<i>P. serratodigitata</i>	Wild		Aril eaten fresh

Table 17.1.1. Continued

Species	Status	Origin	Uses
<i>P. spectabilis</i>	Wild	Amazonian Peru	
<i>P. subpeltata</i>	Wild	Colombia, Venezuela, Mexico, Guatemala, Panama	Fruit eaten fresh, juice
<i>P. tarminiana</i>	Cultivated	Andes from Venezuela to Bolivia	Aril prepared into juices
<i>P. tiliaefolia</i>	Collected	Andes from Colombia to Peru	Aril eaten fresh
<i>P. tolimana</i>	Genetic resource	Colombia	Edible fruit
<i>P. triloba</i>	Wild	Peru, Bolivia	
<i>P. tripartita</i>	Cultivated	Andean Ecuador, N. Peru	Aril eaten fresh
<i>P. tripartita</i>	Cultivated	Andean Venezuela to Bolivia and N. Chile	Aril eaten fresh, juice, sherbets, ice-creams and pastries
<i>P. tripartita</i>	Cultivated	Andes: C. Ecuador to N. Peru	Aril eaten fresh
<i>P. umbilicata</i>	Wild	Argentina	
<i>P. vespertilio</i>	Collected	Trinidad, Guyana, Amazonian Brazil, Peru and Bolivia	Aril eaten fresh
<i>P. villosa</i>	Wild	S. and S.E. Brazil	
<i>P. vitifolia</i>	Collected	Amazonian Venezuela, Brazil, Colombia and Peru	Aril eaten fresh
<i>P. warmingii</i>	Wild	Brazil	

tolerate chilling (Menzel *et al.*, 1990; Gurnah, 1992).

on 'Lacey', 'Purple Gold' and '23-E' (McCarthy, 1995).

1.2. Importance

Passionfruit is grown commercially throughout the tropics and subtropics. Most commercial varieties have been developed based upon selection for superior phenotypes. Brazil is the leading producer, with approx. 44,000 ha devoted to production of the yellow passionfruit, *P. edulis* Sims f. *flavicarpa* Deg. Fruit is consumed fresh or processed for juice, which is exported to the European Union (EU) (Proctor, 1990). Processing utilizes 35% of Brazilian production. In Hawaii, USA, yellow passionfruit production and the juice industry are based on 'Yee Selection', 'Sevcik Selection' and 'Noel's Special' (Ito, 1978; Zee *et al.*, 1995). In Colombia and Venezuela, selections referred to as 'Hawaiana', 'Brasilia amarilla' and 'Brasilia rosada' are grown. In Australia, purple passionfruit is grafted on to yellow passionfruit because of the tolerance of the latter to many root diseases. The Queensland and New South Wales industries are based

1.3. Breeding and genetics

Despite the genetic variability within the *Passiflora* spp., only about 22% of the species have been investigated cytologically (Bowden, 1945; Storey, 1950; Beal, 1969a,b, 1973a,b; Snow and MacDougal, 1993; Melo *et al.*, 2001). According to Beal (1973a) the size of the haploid set ranges from 33 to 70 μm ; there is little variation between the chromosomes and each chromosome is more or less metacentric. The most frequent chromosome numbers are $2n = 2x = 12$ and $2n = 2x = 18$; however, $2n = 14$ (*P. holosericea* L.), 20 and 22 (*P. foetida* L.), 24 and 36 (*P. suberosa* L.) and 84 (*P. lutea* L.) have also been reported (Melo *et al.*, 2001), suggesting that aneuploidy and polyploidy have been important for *Passiflora* evolution. It is assumed that $x = 9$, although the basic chromosome numbers $x = 3$ and $x = 6$ have also been proposed (Storey, 1950; Raven, 1975; Morawetz, 1986; Snow and MacDougal, 1993). The main commercial species and horticultural hybrids of passionfruit are $2n = 18$.

1.3.1. Major breeding objectives

Passionfruit breeding and selection objectives vary with location, but emphasize traits related to fresh fruit production and fruit for the juice industry. Breeding programmes are directed at *P. edulis*, the purple passionfruit, and *P. edulis* f. *flavicarpa*, the yellow form, which has a more vigorous vine and larger fruits. The pulp is less acid and richer in aroma and flavour in the purple, in which a higher proportion of juice (35–38%) is found. For fresh fruit production the criteria include fruit size, firmness, pulp content, shape, colour, flavour and aroma. Yield, acidity and total soluble solids are important for juice production. For both types, the development of cultivars includes the following objectives: (i) yield (productivity, early maturity and self-fertility); (ii) fruit quality (uniform size, yield of pulp and juice, sugar:acid ratio, aroma and colour of pulp, pericarp colour and turgescence); and (iii) disease resistance (Coppens D'Eeckenbrugge *et al.*, 2001).

Most passionfruit selections are susceptible to many pathogens, which severely affect production. The most important diseases include brown spot (*Alternaria* spp.), *Phytophthora* blight (*Phytophthora* spp.), *Fusarium* wilt (*Fusarium oxysporium* f. *passiflorae*) and woodiness virus (PWV). Fruits and leaves are affected by *Alternaria* spp., while *Phytophthora* blight affects the vines, causing defoliation and fruit and root rots. *Fusarium* wilt, which is caused by the soil-borne *F. oxysporium* f. *passiflorae*, can cause devastating losses, which can be controlled by grafting on to *Fusarium*-resistant rootstocks. Symptoms include browning of the root vascular system, the crown and stems. Passionfruit woodiness is aphid-transmitted, and is a serious disease in all production areas, affecting both the purple and the yellow forms (McCarthy, 1995). Susceptibility of diverse *P. edulis* f. *flavicarpa* cultivars to PWV is well documented (Kitajima and Rezende, 2001). Recently, serological and electron microscopy assays have confirmed the presence of PWV infecting *P. nitida* Kunth. in experimental plantings in south-eastern Brazil (Moraes *et al.*, 2002). Another impor-

tant disease is that caused by *Xanthomonas campestris* pv. *passiflorae*, which limits the expansion of the crop in Brazil, because chemical control is ineffective.

Fruit flies are important and widespread, and are difficult to control. Other pests, e.g. the barnacle scale (*Ceroplastes cistudiformis*) and the red-banded thrips (*Selenothrips rubrocinctus*), occasionally increase to serious proportions and cause defoliation (Nakasone and Paull, 1998).

Passiflora germplasm is very diverse, but unexploited. Wild species are being evaluated with the objective of transferring desirable traits into cultivated species. Introgression of resistance genes based on interspecific hybridization between *P. edulis* f. *flavicarpa* and wild *Passiflora* species has been attempted; however, the F₁ hybrids show low fertility (Vieira, 1997). *P. mollissima* Bailey and *P. maliformis* L., for example, are resistant to *X. campestris* pv. *passiflorae*, and *P. giberti* is resistant to *Fusarium* wilt (Oliveira and Ferreira, 1991) and has been used as a rootstock for *P. edulis*. *P. caerulea* has cold tolerance, tolerance of nematodes, collar rot and *Fusarium* wilt (Grech and Rijkenberg, 1991) and tolerance of salinity and poorly drained soils and is used as rootstock for *P. edulis*. *P. manicata*, an Andean species, is a very hardy species, with resistance to nematodes and fungal diseases, e.g. anthracnose, vine mildew and *Alternaria*. It has been used as a rootstock for *P. mollissima* (Coppens D'Eeckenbrugge *et al.*, 2001).

1.3.2. Breeding accomplishments

Scions. Purple tendril colour is dominant over green, and a single recessive gene controls fruit-shell colour, with three phenotypes being recovered in the F₂ population (Nakasone *et al.*, 1967). The inheritance of the self-incompatibility mechanism in the yellow passionfruit has been described as homomorphic sporophytic, and is controlled by two loci (Rego *et al.*, 1999).

In Brazil, the cultivars 'FB-100 – Maguary' (Brix > 14.5°) and 'FB200 – Yellow Master' have been selected for the industry and fresh fruit markets, respectively (Silva, J.R., 1998).

Genetic gains were achieved using commercial orchards as the base populations. 'FB-100 – Maguary' was selected for tolerance of *X. campestris* pv. *passiflora*.

Meletti *et al.* (2000) obtained a series of improved yellow passionfruit selections by recurrent selection. These selections are adapted to south-eastern Brazil and give high yields (45 t/ha per annum). Both purple and yellow passionfruit populations were submitted to a stratified mass selection, in which plants selected in the first cycle were hand-pollinated at the second flowering. Genetic parameters were estimated in a randomized design experiment with 110 clones selected from nine orchards and two replications (Maluf *et al.*, 1989). Clones were grown to maturity and several yield and fruit quality characters were measured. Genetic variation among clones was indicated by large broad-sense heritability for total and early yield (up to 10 weeks) and for fruit weight. These coefficients were moderately high for total soluble solids but subject to considerable genotype $\times$ environment interaction. By visually selecting the 24 highest yielding clones, a genetic gain of 29% was expected for total yield and 89% for early yield, with minor changes in fruit weight, soluble solids and percentage of pulp. Selection among and within half-sib families of yellow passionfruit has been adopted, and 33.7% more fruits have been obtained in comparison to the non-selected progenies. Genetic progress has also been achieved in juice production and total soluble solids (Cunha, 1996, 1999).

In Hawaii (USA), crosses have been made between the *P. edulis* hybrid 'Tom's Special' (field tolerance of leaf and fruit fungal diseases, including *Alternaria alternata*) and three commercial hybrids ('23-E', '3-1' and 'Lacey'). F_1 hybrids were evaluated for seven fruit quality characteristics and disease resistance, of which the best seven were selected for commercial evaluation in comparison with the controls ('23-E' and 'Lacey'). Selections with greater disease tolerance had poorer fruit characteristics. Selections with large fruit, high sugar content (13.8° Brix) and high pulp recovery (50%) were identi-

fied; however, skin colour was poor. F_2 crosses also showed promise (Fitzell *et al.*, 1991).

Rootstocks. In Australia purple passionfruit is grafted on to resistant lines of the yellow form to control soil-borne diseases, e.g. *Phytophthora* blight and *Fusarium* wilt. Oliveira *et al.* (1984) and Menezes *et al.* (1994) evaluated the potential of *P. alata*, *P. caerulea*, *P. edulis*, *P. giberti* and *P. nitida* as rootstocks in Brazil. *P. caerulea* (the blue passionfruit) has been used as a rootstock in Africa (Menzel *et al.*, 1990).

P. quadrangularis, *P. edulis* and *P. edulis* f. *flavicarpa* have been crossed and 27 hybrids were selected in 1996. A further three genotypes were selected under waterlogged conditions as rootstocks resistant to *Fusarium* wilt (Breedt, 1997). According to Yamashiro and Landgraf (1979), *P. alata* could be used to provide resistance to *Fusarium* wilt. *P. edulis* Sims f. *flavicarpa* plants have been grafted on to *P. edulis*, *P. giberti* and *P. cincinnata* (Stenzel and Carvalho, 1992).

2. Molecular Genetics

2.1. Markers

Passionflower classification has been clarified by molecular tools. Isoenzyme variation has been studied in 330 cultivated and wild accessions of *Passiflora*, including the banana passionfruit (*P. mollissima*) and curuba India (*Passiflora* sp.). Polymorphism is greater in the wild species than in the cultivated accessions (Segura *et al.*, 2000). Previously, Do *et al.* (1992) studied the restriction patterns and inheritance of chloroplast DNA (cpDNA) in *P. edulis* and related species. Passion vines collected in the wild resemble *P. edulis* except for the existence of an additional cpDNA fragment. The chloroplast genome size of *P. alata* is close to that of *P. edulis*, which is approx. 110 kb, but *P. alata* appears to be more divergent from *P. edulis* than *P. coccinea*, on the basis of restriction patterns. No fragment similarity has been observed between *P. edulis* and *P. suberosa*.

2.2. Gene isolation and mapping

According to Souza *et al.* (2001), flow cytometry demonstrates that the nuclear DNA content of *P. edulis* is 3.19 pg (2C values). A map-based cloning strategy has potential for identifying passionfruit genes. Linkage maps have been constructed based on molecular markers (Carneiro *et al.*, 2002). A single controlled cross between two clones of *P. edulis* Sims f. *flavicarpa* Deg. ($2n = 18$) was selected for genetic mapping. The parental clones, a commercial variety denoted 123 and a Moroccan introduction denoted 06, are resistant and susceptible to *X. campestris* pv. *passiflora*, respectively, and the passionfruit mapping population was composed of 90 F_1 plants derived from IAPAR 123 (female) $\times$ IAPAR 06 (male). Initially, a total of 380 random 10 bp primers (Operon Tech. Kits OPA, OPAA, OPAB, OPAC, OPAD, OPAE, OPAF, OPB, OPC, OPD, OPE, OPG, OPH, OPI, OPJ, OPM, OPN, OPR and OPT) were screened against the two parents and also a progeny sample composed of six individuals. When a random amplified polymorphic DNA (RAPD) band was present in only one parent and in at least one of the six individuals, the parent was classified as potentially heterozygous for that locus (referred to as a test cross locus). A total of 113 RAPD primers were analysed, according to a two-way pseudo-test cross mapping strategy (Grattapaglia and Sederoff, 1994). Linkage maps were constructed with 269 RAPD markers as on average 2.38 markers per primer show polymorphism between the parents, and also segregated 1 : 1 in the mapping population. For 70% of the markers, the size of the bands ranged from 500 to 1500 bp.

Two separate data sets were obtained, one for each parent. The linkage map for IAPAR 123 consisted of 135 markers (Fig. 17.1.1), with approx. 48% of the markers (65 loci) located on the framework map (Carneiro, 2001). A total of nine linkage groups were assembled covering 728 cM, with an average distance of 11.20 cM between two framework loci. The size of the linkage groups ranged from 56 to 144 cM. The linkage map for the male parent (IAPAR 06) consisted of 96 markers, with 64 loci (approx. 67%) estab-

lishing the framework and covering 783 cM. The average distance between framework loci was 12.20 cM. A total of nine linkage groups were also assembled. The length of the groups ranged from 20 to 144 cM. The estimate for the total genome length (E(G)) was 1195 cM and 1293 cM for the female and male parent, respectively. On average, both maps covered 61% of the *P. edulis flavicarpa* genome (Carneiro *et al.*, 2002). This is the first genetic map in the genus *Passiflora*, which should be a starting point for identifying quantitative trait loci for resistance to *X. campestris* pv. *passiflora*. We have begun to map the same population using amplified fragment length polymorphism (AFLP)-based markers. We have also initiated studies on the response of F_1 plants to bacterial inoculation. The plant response to infection is controlled by oligogenes (Lopes, 2002).

3. Micropropagation

There have been several micropropagation reports involving *Passiflora* (Table 17.1.2). These include clonal propagation from axillary shoots (Moran Robles, 1978, 1979; Drew, 1991; Dornelas and Vieira, 1994; Faria and Segura, 1997a; Passos, 1999) and nodal sections (Kantharajah and Dodd, 1990). Micropropagation has been used for clonal propagation of *Passiflora* species (see Drew, 1997), for *in vitro* germplasm preservation and as a source of protoplasts. In general, Murashige and Skoog (1962) (MS) basal medium supplemented with benzyladenine (BA) has been utilized (Kantharajah and Dodd, 1990; Drew, 1991; Faria and Segura, 1997a; Passos, 1999).

'Norfolk Island' passionfruit was successfully micropropagated on semi-solid MS medium containing 2% sucrose and 8.88 μ M BA for up to 4 weeks, followed by 1–2 weeks on a similar medium without growth regulators and 5 weeks on a root induction medium containing 5.37 μ M naphthalene-acetic acid (NAA). Plantlets were then acclimatized in a humid environment for 4 weeks before transfer to a greenhouse. Plants were ready for field planting after 3 weeks (Kantharajah and Dodd, 1990).

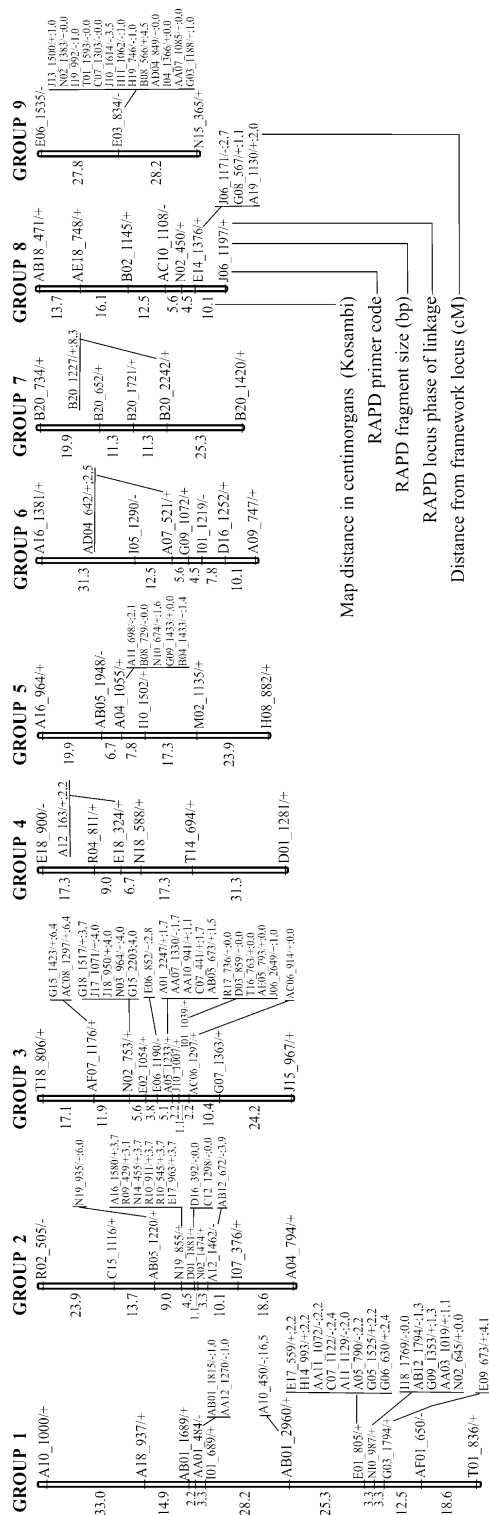


Fig. 17.11. *P. edulis flavicarpa* linkage map.

Table 17.1.2. *Passiflora in vitro* studies (1990 to 2000).

Species	Source of explant	Hormone type and concentration	Reference
<i>P. edulis</i> f. <i>flavicarpa</i>	Internodal segments	4.4–17.7 μ M BA	Biasi <i>et al.</i> , 2000
<i>P. edulis</i> f. <i>flavicarpa</i>	Leaf discs	0.0–5.3 μ M BA	Otahola, 2000
<i>P. edulis</i> $\times$ <i>P. edulis</i> <i>flavicarpa</i>	Cotyledons	10 μ M BA 10% coconut water Transference to 10 μ M NAA	Hall <i>et al.</i> , 2000
<i>P. suberosa</i>	Leaf discs	0.0, 2.2 or 4.4 μ M BA	Monteiro <i>et al.</i> , 2000a
<i>P. caerulea</i>	Leaves	10.0 μ M BA + 0.1 μ M IAA	Jasrai and Mudgil, 1999
<i>P. mollissima</i>	Nodal segments	BA and kinetin at various concentrations and combinations	Cancino <i>et al.</i> , 1998
<i>P. edulis</i> <i>flavicarpa</i>			
<i>P. giberti</i>			
<i>P. edulis</i> f. <i>flavicarpa</i>	Hypocotyl	5 μ M BA + 2 μ M IAA	Faria and Segura, 1997a
	Leaf		
<i>P. edulis</i> f. <i>flavicarpa</i>	Shoot apices	2–20 μ M BA + 2 μ M IAA	Faria and Segura, 1997b
<i>P. foetida</i>	Triploid endosperm	8.8 μ M BA	Mohamed <i>et al.</i> , 1996
<i>P. edulis</i> f. <i>flavicarpa</i>	Shoot primordia	1.0 μ M BA + 1 μ M IBA Transference to 10 μ M BA	Kawata <i>et al.</i> , 1995
<i>P. edulis</i> <i>flavicarpa</i>	Cotyledons	8.88 μ M BA	Dornelas and Vieira, 1994
<i>P. mollissima</i>	Hypocotyledons	10% coconut water	
<i>P. giberti</i>	Leaf		
<i>P. maliformis</i>			
<i>P. amethystina</i>			
<i>P. edulis</i> $\times$ <i>P. edulis</i> <i>flavicarpa</i>	Adult and juvenile buds	10 μ M kinetin + 5 μ M IAA	Drew, 1991
<i>P. edulis</i>	Nodal sections	8.8 μ M BA	Kantharajah and Dodd, 1990

Drew (1991) evaluated various media for micropropagating E23, an F₁ hybrid between *P. edulis* and *P. edulis* f. *flavicarpa*. Growth of nodal buds was promoted by incubation for 1 week in darkness on semi-solid MS basal medium containing 150 μ M N⁶-(2-isopentenyl)adenine (2iP), 200 μ M adenine sulphate and 17.1 μ M indoleacetic acid (IAA). Shoots developed on semi-solid MS medium with 4.9 μ M 2iP and 5.7 μ M IAA. In contrast, juvenile shoots of E23 and *P. edulis* f. *flavicarpa*, *P. edulis*, *P. alata*, *P. caerulea*, *P. mollissima*, *P. coccinea*, *P. herbertiana* and *P. suberosa* responded rapidly on semi-solid MS medium with 10 μ M kinetin and 5 μ M IAA. Proliferation was achieved on MS with 20 μ M BA, 10 μ M kinetin and 5 μ M IAA, and roots were initiated on MS with 5 μ M IAA. These results were confirmed by Dornelas and Vieira (1994) with *P. edulis* f. *flavicarpa*, *P. mollissima*, *P. giberti*, *P. maliformis* and *P. amethystina*.

4. Virus Indexing

Eliminating viruses from clonal passionfruit has been demonstrated. Tissue culture-derived plants of 'Tainong No. 1' (*P. edulis* $\times$ *P. edulis* f. *flavicarpa*) as self-rooted plants, tissue-cultured scions grafted on to seedling *P. edulis* f. *flavicarpa* rootstock and scions grafted on to the same rootstock were evaluated. PWV and passionfruit mottle virus infections were lowest and of shortest duration in the tissue-cultured, own-rooted plants, while infections of grafted plants were the highest (Huang *et al.*, 1997).

5. Somatic Cell Genetics

5.1. Regeneration

5.1.1. Somatic embryogenesis

Anthony *et al.* (1999) reported the establishment of cell suspensions in *P. giberti*, which

apparently had been derived from embryogenic leaf cultures. They reported that early stage somatic embryos appeared on the surface of the cultures. This study has not been verified.

5.1.2. Organogenesis

Induction. Induction of shoot organogenic cultures has been reported for several *Passiflora* species, e.g. *P. amethystina*, *P. caerulea*, *P. edulis* f. *flavicarpa*, *P. foetida*, *P. giberti*, *P. maliformis*, *P. mollissima*, *P. nitida*, *P. quadrangularis* and *P. suberosa*. The anatomy of organogenesis from leaf explants has been described by Appezzato-da-Glória *et al.* (1999).

Organogenesis can occur from callus (Mourad-Agha and Dexheimer, 1979; Scorza and Janick, 1980; Kantharajah and Dodd, 1990; Monteiro *et al.*, 2000a) and directly from the explant (Kawata *et al.*, 1995; Faria and Segura, 1997b; Cancino *et al.*, 1998; Otahola, 2000). Cultures obtained from internodes (Moran Robles, 1978, 1979; Biasi *et al.*, 2000) show callus proliferation and adventitious shoot formation simultaneously. Biasi *et al.* (2000) demonstrated that passionfruit shoot development from explants is asynchronous and continuous. Dornelas and Vieira (1994) reported that 5.37 μM NAA inhibited shoot regeneration on medium containing 17.76 μM BA. Optimum *P. edulis* f. *flavicarpa* shoot regeneration was observed from cotyledon, leaf and hypocotyl explants cultured under light conditions (22 $\mu\text{mol}/\text{m}^2/\text{s}$) on plant growth medium containing at least 4.44 μM BA. Several other authors have also discussed the effect of light irradiation on morphogenic potential of *Passiflora* explants (Moran Robles, 1978; Scorza and Janick, 1980; Kantharajah and Dodd, 1990; Appezzato-da-Glória *et al.*, 1999). Under dark conditions, cotyledon cultures also showed shoot regeneration on medium supplemented with BA, while rhizogenesis occurred in the presence of 5.37 μM NAA.

Plant regeneration without a callus phase has been demonstrated with *P. mollissima* and has been optimized for *P. edulis* f. *flavicarpa*

and *P. giberti*. Leaf and root segments from 4- to 5-week-old axenic nodal segment-derived plants were used for regeneration studies. Semi-solid MS medium supplemented with BA and kinetin was used to induce shoots (Cancino *et al.*, 1998).

Monteiro *et al.* (2000a) reported BA-induced callus induction on *P. suberosa* leaf discs, with organogenesis occurring only after transfer to MSM medium (Monteiro *et al.*, 2000b) supplemented with gibberellic acid (GA_3). Coconut water causes increased *P. quadrangularis* cell proliferation (Mourad-Agha and Dexheimer, 1979) and shoot formation of *P. edulis* (Kantharajah and Dodd, 1990). According to Dornelas and Vieira (1994), at least half of *Passiflora* cultures derived from cotyledons form shoots on MS medium containing 8.88 μM BA and 10% (v/v) coconut water. The percentages of explants that produced shoots were 56 in *P. giberti*, 70 in *P. amethystina* and *P. mollissima*, 90 in *P. maliformis* and 96 in *P. edulis flavicarpa*, in which the mean number of shoots for each explant type was 57.3, 18.2 and 46.0, respectively, for cotyledon, hypocotyledon and leaf tissues. Faria and Segura (1997b) and Marota *et al.* (2001) demonstrated that 8.8 μM silver thiosulphate (STS) significantly increases the differentiation and development of passionfruit adventitious shoots derived from hypocotyls and leaf explants by reducing ethylene in the culture vessels.

Development. Shoot elongation and rooting is initiated on half-strength MS medium. Plantlet survival under greenhouse conditions depends on the presence or not of leaflets on the regenerants (Dornelas and Vieira, 1994).

5.1.3. Triploid recovery

In vitro culture of endosperm has been utilized to regenerate triploid plants when seediness is undesirable and unnecessary. Mohamed *et al.* (1996) described the development of triploid *P. foetida* plants from endosperm halves after removal of zygotic embryos. Regeneration was observed on semi-solid MS media containing: (i) 5 μM

NAA, 2 μ M BA and 250 mg/l peptone; and (ii) 29 μ M GA₃ + 1 g/l casein hydrolysate, although the controls also showed regeneration.

5.1.4. Protoplast isolation and culture

Protoplast-to-plant technology has been described for passionfruit (Manders *et al.*, 1991; Dornelas and Vieira, 1993; d'Utra Vaz *et al.*, 1993; Dornelas *et al.*, 1995; Otoni *et al.*, 1995; Vieira and Dornelas, 1996; Anthony *et al.*, 1999). According to Dornelas and Vieira (1993), *Passiflora* seeds are surface-disinfested before germination on half-strength MS medium. Cotyledon tissues are removed from 15-day-old seedlings and cut transversely into 1 mm slices. Tissues are plasmolysed for 20 min in CPW13 (Frearson *et al.*, 1973) containing 5 mM methyl ethane sulphonate (MES) and 13% (w/v) mannitol (pH 5.8). CPW13 is replaced with 5 ml of enzyme mixture, and cultures are incubated in darkness for 16 h on a shaker at 30 rpm. The released protoplasts are filtered through filtration fabric (64 μ m), pelleted by centrifugation (1500 rpm for 7 min) and rinsed in CPW13. The enzyme mixture containing 2.0% cellulase and 0.4% Macerozyme (Onozuka, Yakult) produced 4.0×10^6 protoplasts per half a gram of green tissue.

Protoplasts from pollen grains of *P. edulis* f. *flavicarpa* and *P. maliformis* and from tetrads of *P. edulis* f. *flavicarpa*, *P. incarnata* and *P. alata* can also be isolated but this requires a germination step, and yield is associated with pollen germination ability. On average, 5.4×10^4 and 1.2×10^5 protoplasts have been obtained from germinated pollen grains and from microspores, respectively, at the tetrad stage. The enzyme cellulysin, which contains xylanases, glucanases, pectinases and chitinases, is essential (Dornelas *et al.*, 1995).

Cotyledon-derived protoplasts are cultivated at 1×10^5 cells/ml of liquid K8P medium (Kao and Michayluk, 1975; Gilmour *et al.*, 1989) or embedded in 0.6% (w/v) agarose. K8P medium (3 ml) is also used to bathe the embedded protoplasts. By day 7, 600 μ l (approximately a quarter of the liquid volume) of K8P medium is added to the

plates. For reducing osmotic pressure, a quarter of the liquid volume is replaced by a mixture of K8P and K8 (2 : 1) on the 14th day of culture. The same procedure is used on the 21st day and after 1 month.

Protoplast-derived colonies (1 mm diam.) are transferred to 1.5% (w/v) Phytagel-solidified MD medium. After 3 to 4 weeks, microcalluses are transferred to light (25–50 μ mol/m²/s) and placed on MS medium supplemented with 8.88 μ M BA, 10% (v/v) coconut milk and 3% (w/v) sucrose for shoot regeneration. Manders *et al.* (1991) and d'Utra Vaz *et al.* (1993) observed that the transfer of leaf-derived colonies to MS containing 26.85 μ M NAA and 1.11 μ M BA under light conditions produces calluses that could be transferred into regeneration medium (MS with 4.44 μ M BA) within 30 days. Regeneration of two to three shoots per callus was observed from 40% of the protoplast-derived calluses after 90 days.

Dornelas and Vieira (1993) also cultivated freshly isolated protoplasts of *P. edulis* f. *flavicarpa*, *P. amethystina* and *P. cincinnata* on medium described by Manders *et al.* (1991). Shoot regeneration was noted after 50 days in culture (Fig. 17.1.2). According to the species, 50 to 90% of the calluses produced shoots, and a mean of 12 plantlets per callus was observed for *P. edulis* f. *flavicarpa*.



Fig. 17.1.2. Regeneration of *P. amethystina* Mikan from protoplast culture.

P. edulis f. *flavicarpa* plants have reached maturity in the field, with normal flowers and fruit set.

5.2. Genetic manipulation

5.2.1. Somatic hybridization

Breeding objectives. Somatic hybrids could be used as rootstocks for protecting passion vines against soil-borne diseases caused by *Fusarium* and *Phytophthora*. Dornelas *et al.* (1995) produced somatic hybrids ($4n = 4x = 36$) of *P. edulis* f. *flavicarpa* ($2n = 2x = 18$) and four wild diploid species ($2n = 2x = 18$) in order to introgress alien traits into passion-fruit, namely *Fusarium* wilt resistance from *P. alata* and *X. campestris* pv. *passiflora* resistance from *P. cincinnata*.

Protocol. Protoplasts of the cultivated species were isolated from leaf tissues of *in vitro* plants, while protoplasts of the wild species were isolated from callus-derived suspension cultures. Callus from leaf discs was induced on semi-solid MD medium in the dark, and suspensions were established in Erlenmeyer flasks containing liquid MD on an orbital shaker at 130 rpm. After washing and centrifugation, parental protoplasts were suspended and polyethylene glycol (PEG)-mediated chemical fusion was used. The fusion protocol (Power and Chapman, 1985) included an initial adjustment of the parental protoplasts to a density of 2×10^5 protoplasts/ml of CPW13 solution. The protoplasts were dispensed as follows: 2 ml of *P. edulis* plus 2 ml of wild *Passiflora* (two tubes), 2 ml of *P. edulis* (one tube for viability control) and 2 ml of wild *Passiflora* (one tube for viability control). After spinning the tubes (1500 rpm, 7 min) CPW13 solution was removed, and 1 ml of the fusion agent was added per tube (30% PEG 6000, 4% sucrose, 0.01 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH 5.7). The protoplasts were incubated for 10 min. The PEG solution was subsequently diluted at 5 min intervals by adding the following volumes of CPW13: 0.25, 0.5, 1.0, 1.0 and 1.5 ml. Protoplasts were washed twice by resuspension in CPW 13 and centrifuged. On average, 9 to 13% of the

protoplasts involved in fusion are heterodimers.

The products of the fusion experiments are cultured in 0.6% agarose droplets in K8P medium, replacing the bathing medium every week. Regeneration of cell walls and presence of binucleate cells (8.7–15.7%) were observed after 36 h. Microcolonies (28-day-old colonies with 1 mm diam.) are transferred to solid MD medium after 2 weeks. Plates are maintained in darkness for 60 days for development. At this stage, the selection of putative somatic hybrids can be performed.

Soluble protein analysis was used to verify hybrid calluses. We have identified 4.5% of hybrid calluses for *P. edulis* (+) *P. alata* and 3.4, 3.6 and 5.3% for *P. edulis* (+) *P. amethystina*, *P. edulis* (+) *P. cincinnata* and *P. edulis* (+) *P. giberti* (Fig. 17.1.3), respectively. Band patterns of three isozyme systems (esterase, malate dehydrogenase and peroxidase) used to confirm these results provided further evidence of the hybrid nature of the calluses. Selected microcalluses were placed on MS + 4.44 μM BA supplemented with 5% (v/v) coconut milk and 3% (w/v) sucrose, and plates were incubated in the light. The frequency of shoot formation was approximately 96, 63, 98 and 83% for *P. edulis* (+) *P. alata*, *P. edulis* (+) *P. amethystina*, *P. edulis* (+) *P. cincinnata* and *P. edulis* (+) *P. giberti*, respectively. Root formation was induced in half-strength MS medium devoid of hormones, and these shoots developed into whole plants. The hybrid nature of regenerants was confirmed by chromosome counts of cells in the root tips ($2n = 4x = 36$). Somatic hybrid plants have been transferred to the field.

Accomplishments. Barbosa and Vieira (1997) studied the diploid parents *P. edulis* f. *flavicarpa* and *P. amethystina* (both $2n = 18$) and the somatic hybrid plants ($4n = 36$) denoted (E + Am) no. 12, (E + Am) no. 13, (E + Am) no. 28 and (E + Am) no. 35 produced by Dornelas *et al.* (1995). The morphology of the hybrids was intermediate with respect to leaf and flower characteristics. Most of the materials showed high levels of pollen viability. Significant differences in pollen grain viability between the parental species and

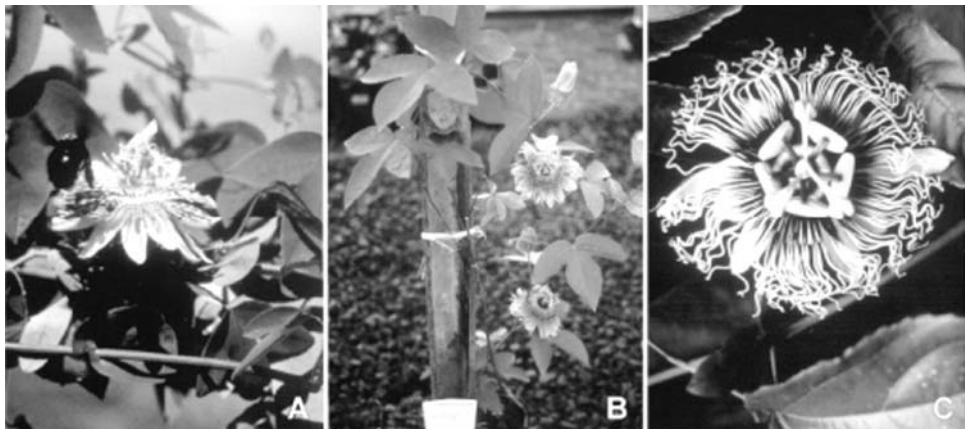


Fig. 17.1.3. *P. giberti* (A), *P. edulis* f. *flavicarpa* (C) and the somatic hybrid (B).

the somatic hybrids were detected. The correlation between mean pollen viability and meiotic irregularities was high and negative.

The analyses for hybrid plants produced by protoplast fusion between *P. edulis* f. *flavicarpa* and *P. cincinnata* are summarized in Table 17.1.3. Those somatic hybrids were $4n = 36$, with no occurrence of aneuploids. Four nucleolar organizer regions were observed, confirming the hybrid nature of

the plants. The simplest interpretation of the global pattern of segregation suggests that the quadrivalents may correspond to the two pairs of parental chromosomes presenting nucleolar-organizing sites. The amounts of pollen produced by the hybrids have encouraged us to use them as a male parent in back-crossing programmes. These results indicate stable meiotic behaviour in *P. edulis* f. *flavicarpa* + *P. cincinnata* somatic hybrids. Pollen viability (Table 17.1.4)

Table 17.1.3. Meiotic behaviour of four somatic hybrid plants obtained by protoplast fusion between *P. edulis* f. *flavicarpa* and *P. cincinnata* with respect to prophase I figures. Minimal number of cells analysed = 40.

Hybrid plants	16 II + 1 IV	2 I + 17 II	18 II
E + C no. 7	30.0	15.0	55.0
E + C no. 14	17.1	4.3	78.6
E + C no. 25	13.3	15.0	71.6
E + C no. 26	32.5	7.5	60.0

Table 17.1.4. Pollen viability data (in the diagonal) in the parental forms and four somatic hybrid plants obtained by protoplast fusion between *P. edulis* f. *flavicarpa* and *P. cincinnata*, *t* test analysed (*5%, **1% of probability).

	(E)	(C)	E + C no. 7	E + C no. 14	E + C no. 25	E + C no. 26
<i>P. edulis flavicarpa</i> (E)	96.12 ± 2.06	0.65ns	17.77**	20.57**	24.53*	13.98**
<i>P. cincinnata</i> (C)		96.77 ± 0.93	18.42**	21.22**	25.18**	14.63**
E + C no. 7			78.35 ± 7.53	2.80ns	6.76**	3.79ns
E + C no. 14				75.55 ± 5.09	3.96*	6.59*
E + C no. 25					71.59 ± 2.06	10.55**
E + C no. 26						82.14 ± 7.60

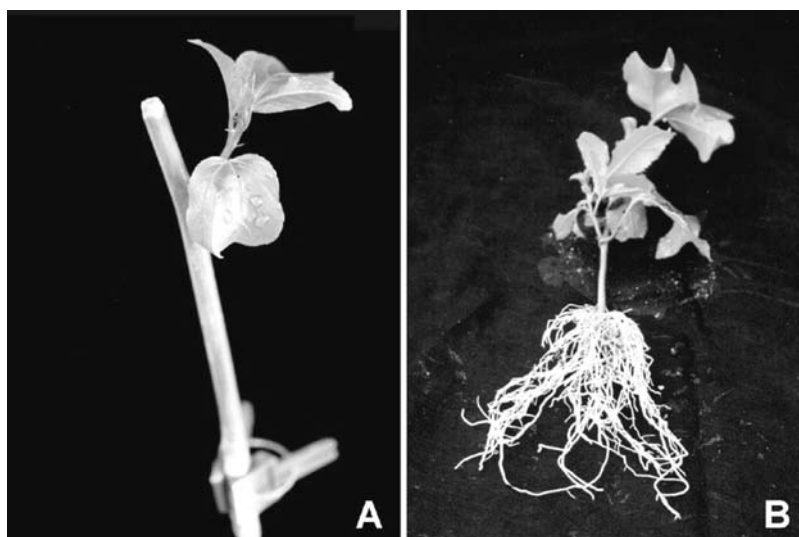


Fig. 17.1.4. Grafting *P. edulis* f. *flavicarpa* on to a *P. edulis* f. *flavicarpa* + *P. cinninnata* rootstock (A), showing a well-developed rooting system after 40 days (B).

supports the use of these materials as a bridge for the genetic improvement of yellow passionfruits for the introgression of genes, as *P. cinninnata* is resistant to *X. campestris* pv. *passiflorae*. These hybrid plants are also being tested as rootstocks for *P. edulis* *flavicarpa* (Fig. 17.1.4).

5.2.2. Genetic transformation

Breeding objectives. Genetic transformation is an important strategy for *P. edulis*, since PWV and *X. campestris* pv. *passiflorae* are the major limiting factors for this crop.

Protocol. Transient gene expression in *P. edulis* mediated by *Agrobacterium tumefaciens* has been reported (Cancino *et al.*, 1998; Silva, M.B., 1998; Hall *et al.*, 2000). A supervirulent *A. tumefaciens* strain 1065, carrying both the β -glucuronidase (*uidA* or GUS) and neomycin phosphotransferase (*nptII*) genes, has been used to infect leaf and root segments of *P. edulis*, *P. mollissima* and *P. giberti*. Several factors, including plant genotype, explant type, bacterial dilution, inoculation and co-cultivation times, were evaluated in relation to transient *uidA* gene expression. The effect of

sonication in conjunction with *Agrobacterium*-mediated transformation has a positive effect on transient gene expression with *P. edulis* and *P. mollissima* (Cancino *et al.*, 1998). Manders *et al.* (1994) utilized the GV3111SE strain of *A. tumefaciens* and the pMON200 vector that contains the *nptII* gene, and obtained three transformed shoots.

Stable transformation of passionfruit with *nptII* and *uidA* has been described by Silva, M.B. (1998), who used the LBA4404 strain in *Agrobacterium rhizogenes* MAFF03-01724. The bacteria were smeared over a stem segment of *P. edulis* on MS semi-solid medium. Tumours appeared after 4 weeks. Adventitious roots derived from tumours were removed and cultured on medium with claforan, an antibiotic. The hairy roots were transferred to medium supplemented with BA and 2,4-D, where they developed into plantlets (Lin, 1998).

With yellow passionfruit, the biolistic approach was tested in order to transfer the bactericide *attacinA* gene driven by the 35S–35S promoter to plants of an elite cultivar (Vieira *et al.*, 2002). The objective is to produce transgenic plants resistant to *X. campestris* pv. *passiflorae*. Selective agent sensitivity was evaluated, and 5 mg/l hygromycin inhibited

organogenesis in 60% of the explants. Kanamycin at 50 mg/l was also effective. Transient gene (*uidA*) expression was effective in the following conditions: 1200 psi of helium pressure and 9.5 cm flight distance. Co-transformation frequencies of 0.6% were obtained, and transgene insertion was confirmed by polymerase chain reaction (PCR) for both GUS and *attacinA*.

Accomplishments. Braz (1999) introduced a sequence derived from the replicase and capsid protein (cp) genes isolated from PWV. Two-thirds of the 3' region of the replicase gene and the 5' region of the cp gene were fused. This cassette does not contain the start codon, i.e. resistance is mediated by post-transcriptional silencing after hybridization between the viral RNA and the RNA produced by the transgenic plant. Preliminary results suggest that this strategy can be used to control the virus disease.

5.3. *In vitro* preservation

In vitro conditions have been optimized for maintaining a *Passiflora* collection in our laboratory. We are maintaining 20 species as shoot cultures at $26 \pm 2^\circ\text{C}$, 16 h light ($22 \mu\text{mol}/\text{m}^2/\text{s}$). Shoots are individually kept in flasks ($8 \times 15 \text{ cm}$, 30 ml) on half-strength

MS medium, free of hormones, which is replaced at 45–60-day intervals.

6. Conclusions

The major applications of *in vitro* techniques to passionfruit include the preservation of genetic resources and the recovery of transgenic plants, although further work is required to establish protocols for somatic embryogenesis. There are several horticultural traits that can be altered using genetic engineering approaches, i.e. resistance to woodiness disease and fungal and bacterial diseases and improved fruit quality and yield. Molecular markers can aid in the mapping, cloning and sequencing of important genes.

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18

Rosaceae

The family *Rosaceae* consists of trees, shrubs and mainly perennial herbs, with species ranging geographically from the tropics to the Arctic. There are four subfamilies: *Spiraeoideae*, *Rosoideae* (e.g. *Rosa* spp., *Rubus* spp., *Fragaria* spp., etc.), *Amygdaloideae* or *Prunoideae* (e.g. *Prunus* spp., etc.) and *Maloideae* (e.g. *Malus* spp., *Eriobotria japonica*, *Pyrus* spp., *Cydonia* spp., etc.) (Watson and Dallwitz, 1992 onwards). It is estimated that there are approx. 100 genera and 2000 species. Most species within the family have flattened flowers with five sepals and five

petals with wavy margins, and ten or more stamens. In the *Rosoideae* many apocarpous pistils develop into achenes. In the *Prunoideae* a single monocarpellate pistil matures into a drupe. In the *Spiraeoideae* the gynoecium consists of two or more apocarpous pistils that develop into follicles. Except for the *Maloideae*, the ovary is superior and there is usually some development of a perigynous zone. Within the *Maloideae*, the ovary is compound and inferior, and an epigynous zone can occur.

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<http://biodiversity.uno.edu/delta/>

18.1 *Fragaria* Strawberry

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1. Introduction

1.1. Botany and history

Strawberry belongs to the genus *Fragaria*, tribe *Potentilleae* of the *Rosaceae* family. The 19 or so wild species of *Fragaria* have four naturally occurring chromosome groups: diploid, tetraploid, hexaploid and octoploid. All early accounts of the strawberry refer to the wild woodland species *F. vesca* L. of Europe, which is small fruited (Turner, 1548). The cultivated strawberry *Fragaria* × *ananassa* (Duch.) is considered to have arisen in France in the 18th century from a chance cross involving two native American species, the North American or meadow strawberry *F. virginiana* (L.) Duch. with the Chilean strawberry, *F. chiloensis* Duch. (Duchesne, 1766; Fletcher, 1917). *F. virginiana* is found across most of the USA and into Canada, whereas *F. chiloensis* is restricted to a narrow coastal zone along the Pacific coast of the Americas (J.F. Hancock, personal communication). Two other species, *F. vesca*, the most widely distributed wild species, and *F. moschata* Duch., are also grown commercially, but on a much smaller scale (Hancock *et al.*, 1992). Unlike *F. vesca*, which is diploid ($2n = 2x = 14$), both *F. virginiana* and *F. chiloensis* are octoploid ($2n = 8x = 56$), and it is at this level of ploidy that nearly all of the hybridization and selection has been performed to produce the high-yielding and highly adapted modern cultivars.

The plants are low growing, spreading with runners, which are able to root to produce new plants. The crown of the plant produces shoots, which flower and fruit. The juicy edible fruit is an enlarged receptacle on whose surface seeds are embedded. Outcrossing is the common method of strawberry breeding, with segregation for most characters occurring in each generation (Darrow, 1966). The modern cultivars have hermaphrodite flowers and therefore must be emasculated for controlled cross-pollinations. The blossom is composed of many pistils, each with its own style and stigma, attached to a receptacle, which develops into a fleshy fruit after fertilization. Seed germination is variable with respect to time and the percentage of germination from each family. After seedlings do emerge, they are very tender during the first few weeks. Strawberry selections and cultivars are propagated vegetatively by runner plants rather than by seed (Scott and Lawrence, 1975).

The earliest cultivars resulted from the selection of superior phenotypes from chance crossings, but by 1817 Thomas Knight in England was raising seedlings from controlled crosses and released the cultivars 'Downton' and 'Elton'. 'Keen's Seedling', named in 1819 and raised by Michael Keen, a market gardener from London, and 'Downton' were widely used as parents in strawberry breeding (Scott and Lawrence, 1975).

1.2. Importance

Recent statistics on world production of strawberries (Hancock, 1999) indicate that they are the most important soft fruit worldwide. Approximately 25% of world strawberry production is concentrated in North America (Hancock and Bringham, 1988) with 85% of this in California, USA. In the European Union (EU), Spain and Italy account for approximately 50% of production. Japan also has a substantial strawberry industry, which is second only to that of the USA (Oda, 1991). Current world production of strawberry is 3,198,689 t (FAOSTAT, 2004).

1.3. Breeding and genetics

1.3.1. Major breeding objectives

Modern breeding is largely based upon the identification of superior phenotypes, hybridization among these, followed by selection of the best offspring for trials as potential cultivars and/or their use as parents for the next generation. *Fragaria* × *ananassa* is very heterozygous (Scott and Lawrence, 1975), and suffers from a rapid loss of vigour, yield and fruit size with inbreeding (Morrow and Darrow, 1941, 1952; Spangelo *et al.*, 1971). Selection and, to a lesser extent, the use of inbreeding have resulted in a narrow germplasm base (Darrow, 1966; Sjulín and Dale, 1987). Lack of genetic diversity can result in vulnerability to environmental stresses. In general, all major strawberry cultivars have arisen from common ancestors (Scott and Lawrence, 1975). Utilizing the diverse array of germplasm that exists in strawberry both from regionally adapted cultivars and also from exotic sources is essential for future breeding.

Most breeding programmes have similar objectives, but the relative importance of these objectives depends on the regional climate and targeted sector. In all programmes, fruit quality, yield and pest and disease resistances (Scott and Lawrence, 1975) are of major importance. In terms of fruit quality,

size, flavour (especially sweetness), firmness and colour, followed by other factors, e.g. skin strength, are all important for consumer acceptance. Addressing pest and disease problems, which hinder production and have an effect on consumer acceptance, is also vital to the breeder. The major pests of strawberry include aphids, root weevils and mites. A number of pathogens have become of great concern, e.g. red core or red stele caused by *Phytophthora fragariae* Hickman, *Phytophthora cactorum* (Leb. and Cohn) Schroet., *Verticillium* wilt (*V. albo-atrum* Reinke & Berth. and *V. dahliae* Kleb), anthracnose caused by *Colletotrichum* spp. and *Botrytis cinerea*. For a review of the major pest and disease problems of strawberry and the genetic resources to combat these, see Hancock *et al.* (1992). Vigour, fruiting habit, time to ripen, winter hardiness and blossom frost hardiness are also important objectives. The success of strawberry cultivars is greatly influenced by the interaction of a wide range of environmental and plant genetic factors, e.g. photoperiod, temperature, disease resistance, tolerance of different soil conditions, winter hardiness, high-temperature tolerance and inherent vigour (for more details see Hancock *et al.*, 1992).

1.3.2. Breeding accomplishments

Strawberry is one of the few fruit crops that is characterized by a steady replenishment of new and improved cultivars (Janick, 1997). Due to extensive breeding work utilizing the diverse range of available germplasm, it is now possible to grow strawberries in many different climates, from the temperate Mediterranean to subtropical and taiga zones (Hancock *et al.*, 1992). Galletta *et al.* (1981) listed 19 breeding programmes in the USA alone, and a more recent overview of the US Department of Agriculture (USDA) strawberry programme has been provided by Galletta *et al.* (1997). For details of breeding programmes in Europe, see Rosati (1991), Simpson (1991) and Roudeillac (2000). Information on strawberry germplasm can be found in Hummer and Strik (1997), Khanizadeh and Prasher (1997) and Khanizadeh *et al.* (1999).

The extent of genetic diversity available in strawberry cultivars has been examined. Sjulín and Dale (1987) studied the pedigrees of 134 strawberry cultivars that had been released since 1960 and found that they could be attributed to 52 founding clones. Graham *et al.* (1996) compared estimates of genetic diversity from a range of strawberry cultivars using both pedigree analysis and molecular markers, and demonstrated the closely related nature of strawberry cultivars, despite their having been produced from geographically distinct breeding programmes. Consequently, the successful development of desirable combinations of traits in future cultivars may be limited by a lack of genetic diversity. In order to address this problem, the number of genetically diverse parents in each generation can be expanded. In addition, the inclusion of unrelated *Fragaria* × *ananassa* germplasm from other breeding programmes and germplasm from wild *Fragaria* species (Luby *et al.*, 1991; Hancock *et al.*, 1992) could be used to expand the genetic base of cultivars. For example, genes that determine cyclic flowering in all commercial strawberry cultivars (*Fragaria* × *ananassa* Duch.) have been derived from a single source of *F. virginiana* ssp. *glauca* from the Wasatch Mountains in Utah, USA. To broaden the germplasm base of cyclic flowering cultivars, the reproductive characteristics of five to ten colonies of *F. virginiana* ssp. *glauca* have been examined (Sakin *et al.*, 1997).

2. Molecular Genetics

2.1. Gene cloning

Several strawberry genes have been isolated and cloned, particularly those genes involved in fruit ripening. Strawberry is a non-climacteric fruit (Davies, 1987), in which ripening is a complex developmental process that involves many changes in gene expression. In climacteric fruits, these events are coordinated by ethylene, which is synthesized autocatalytically in the early stages of ripening. Non-climacteric fruits do not synthesize or respond to ethylene in this manner

and yet undergo many of the same physiological and biochemical changes associated with ripening.

Manning (1997, 1998) identified 66 differentially expressed clones by screening a complementary DNA (cDNA) library prepared from ripe strawberry. The partial sequences of these cDNAs were compared with database sequences, and 26 families of non-redundant clones were identified. These sequences, several of which are novel to fruits, encoded proteins involved in key metabolic events, including anthocyanin biosynthesis, cell wall degradation, sucrose and lipid metabolism, protein synthesis and degradation and respiration. Nam *et al.* (1999) used wild strawberry (*F. vesca*) as a model for studying ripening because of its small diploid genome, its short reproductive cycle and its capacity for transformation. Eight ripening-induced cDNAs from this species were isolated after differential screening of a cDNA library. The predicted polypeptides of seven of the clones exhibit similarity to database protein sequences. A ninth cDNA clone was constitutively expressed and predicted to encode a metallothionein-like protein. None of these proteins appeared to be directly related to events generally associated with ripening; rather, their putative functions were indicative of the wide range of processes up-regulated during fruit ripening.

Expression of endo- β -1,4-glucanases in the ripening process has been studied. Llop-Tous *et al.* (1999) isolated two endo- β -1,4-glucanase cDNA clones (Cel1 and Cel2) from a cDNA library obtained from ripe strawberry fruit. Northern analysis showed that both genes were highly expressed in fruit and that they had different temporal patterns of accumulation. Harpster *et al.* (1998) studied the expression of a ripening-specific, auxin-repressed endo- β -1,4-glucanase gene in strawberry. In fruit, Cel1 mRNA was first detected at the white stage of development, and at the onset of ripening, coincident with anthocyanin accumulation.

Expansins, cell wall proteins thought to disrupt hydrogen bonds within the cell wall polymer matrix, thereby aiding the process of tissue softening, have also been cloned.

Civello *et al.* (1999) isolated a full-length cDNA encoding an expansin gene expressed in ripening strawberry fruit.

A cDNA clone encoding a putative dihydroflavonol 4-reductase gene was isolated from a 'Chandler' DNA subtractive library (Moyano *et al.*, 1998). On the basis of Northern analysis, they proposed that the putative dihydroflavonol 4-reductase gene is involved in the biosynthesis of anthocyanin during colour development at the late stages of strawberry fruit ripening. During the first stages, the expression of this gene could be related to the accumulation of condensed tannins.

A cDNA from a strawberry fruit subtractive library with homology to class I low-molecular-weight heat-shock protein genes from other higher plants has been isolated (Medina-Escobar *et al.*, 1998). This gene is not expressed in roots, leaves, flowers and stolons, but is expressed in fruits at specific stages of elongation and ripening; however, a differential pattern of mRNA expression was detected in the achenes and receptacle.

Wilkinson *et al.* (1995) and Hamano *et al.* (1998) used the differential display technique to study ripening. Wilkinson *et al.* (1995) identified five mRNAs with ripening-enhanced expression. Three of the mRNAs were fruit-specific, with little or no expression detected in vegetative tissues. Sequence analysis of cDNA clones revealed positive identities for three of the five mRNAs based on homology to known proteins. Hamano *et al.* (1998) identified 11 polymerase chain reaction (PCR) products related to differential gene expression during fruit development. Two cDNAs of the 11 PCR products were subcloned and sequenced. One clone had homology to the S6 kinase homologue of *Arabidopsis thaliana* and the other to the gene that encodes hydroxyproline-rich glycoprotein.

Several other genes have been identified. Ndong *et al.* (1997) identified several cDNAs showing differential expression at low temperature by differential screening of a cDNA library prepared from cold-acclimatized strawberry plants. Orthologues of *CBF1*, a cold-induced transcription factor from *Arabidopsis*, have been cloned from

strawberry using degenerate PCR primers (Owens *et al.*, 2002b).

Screening of genomic libraries with known cDNAs from other species has been used to identify genes with high similarity in strawberry. Lazarus and Macdonald (1996) isolated a gene encoding an auxin-binding protein (ABP1) from strawberry by screening a genomic library with an ABP1 cDNA from maize. Reverse transcription (RT)-PCR was used to amplify the complete coding region for cloning as cDNA, and a recombinant baculovirus was constructed for the expression of strawberry ABP1 in insect cells.

Kim and Chung (1998) isolated a genomic DNA harbouring a cytosolic ascorbate peroxidase gene (ApxSC) from a genomic library of the strawberry (*Fragaria* × *ananassa*).

With the development of high-throughput genomics techniques (see Section 2.3) the pace of identification and cloning of genes should increase significantly.

2.2. Markers and marker-assisted selection

Initially, genes were used as markers; however, only a limited number of these are distinguishable on the basis of phenotype. Isozymes have also been studied in *Fragaria*; however, they are of limited value due to the low numbers available and difficulty in saturating a genetic map (Arulsekar and Bringhurst, 1983; Williamson *et al.*, 1995). Other marker systems that exploit polymorphisms at the DNA level are much more abundant and informative. Restriction fragment length polymorphisms (RFLPs) are DNA fragments that vary in length due to mutations in restriction sites (Botstein *et al.*, 1980). The inheritance of the DNA sequence variations (or alleles) can be followed in the same way as conventional markers. RFLPs are co-dominant locus-specific markers; however, they are technically difficult and time-consuming to develop and they require radioisotopes for detection. Randomly amplified polymorphic DNAs (RAPDs) provide a technically simpler marker for mapping and marker-assisted selection (MAS) (Williams *et al.*, 1990). Amplified fragment

length polymorphisms (AFLPs) are restriction fragments generated by digesting genomic DNA and identified by selective amplification. Although more numerous, they also have technical limitations (Vos *et al.*, 1995). RAPDs and AFLPs are dominant so that complete determination of genotypes in a segregating population is impossible. RAPDs and AFLPs can be converted to co-dominant markers by cloning, sequencing and conversion to a locus-specific PCR-based assay.

Accurate identification of strawberry species and cultivars, better classification of species material and better understanding of the genetic relationships within and between species would allow more effective use of germplasm in breeding programmes. Cultivar identification is difficult on the basis of morphological characters alone due to the variability of traits within a cultivar and high levels of similarity among cultivars (Nielsen and Lovell, 2000). When based solely on vegetative characters, accurate identification is almost impossible, and for a clonally propagated crop this causes serious problems. RAPD analysis has been used successfully to identify and differentiate several strawberry cultivars, including closely related ones (Gidoni *et al.*, 1994; Parent and Page, 1995; Graham *et al.*, 1996; Landry *et al.*, 1997; Degani *et al.*, 1998). AFLP markers have also been used to fingerprint strawberry cultivars, although a better correlation with the coefficients of co-ancestry has been observed with RAPD marker data (Degani *et al.*, 2001). Classification of varieties using inter-simple sequence repeat (ISSR) markers is consistent with the pedigree data and broadly comparable with the classification obtained from AFLPs (Arnau *et al.*, 2001).

Marker studies have also attempted to resolve genetic relationships within and between strawberries. Wild strawberry and wild strawberry-like species of northwestern Argentina have been studied (Ontivero *et al.*, 2000). Relationships among North American octoploid strawberry populations have been studied by evaluating morphological traits and RAPD markers (Harrison *et al.*, 1997b). RAPD data defined three groups: (i) *F. virginiana* ssp. *virginiana* and ssp. *glauca*; (ii) *F.*

chiloensis; and (iii) *F. virginiana* ssp. *platypetala*. The latter is more similar to *F. chiloensis* than to *F. virginiana*, suggesting it is probably a subspecies of *F. chiloensis*. Harrison *et al.* (1997b) concluded that all octoploid North American strawberries may have a common ancestor and have differentiated into *F. chiloensis* and *F. virginiana* by adapting to moister and drier environments, respectively. Porebski and Catling (1998) examined intraspecific classification of *F. chiloensis*, including North American ssp. *lucida* and *pacifica* and South American ssp. *chiloensis* using RAPDs. Chloroplast DNA restriction fragment variation has been examined among strawberry taxa (Harrison *et al.*, 1997a). Other species have been examined by comparative sequencing of SI nucle-ase-digested long-range PCR products from mixed-template amplifications. *F. moschata* resembles *F. viridis*, but differs from *F. vesca*, *F. nubicola* and a closely related out-group representative, *Duchesnea indica* (Lin and Davis, 2000). The information generated by these marker techniques is being applied in breeding programmes for selection of parents and utility of species for introgression.

MAS can be used to enhance plant breeding by selection of plants with desired trait(s) accurately and at an early stage. Markers tightly linked to the gene of interest can be screened rather than the trait itself. Markers linked to a particular trait can be identified through the construction of a linkage map in a population segregating for the trait of interest. Alternatively, bulked segregant analysis can be used to identify markers linked to a particular trait whose position can then be determined on a linkage map. To map a new gene it is necessary to have a large number of different markers spaced along each chromosome and test for co-segregation of this gene with each marker. The greater the number of markers available for each chromosome the more useful will be the mapping data.

With the development of genomics or high-throughput genetics involving large-scale gene or DNA sequence identification the development of other marker types such as simple sequence repeats (SSRs) can be achieved quickly for MAS (Beckman and

Soller, 1990). A significant part of the genome is made up of these SSRs, in which a short motif is repeated in tandem. SSRs are variable in the number of repeats they contain due to slippage during DNA replication and are dispersed throughout the genome, and therefore are useful as markers. After SSRs are identified, PCR primers can be designed to the flanking sequences, allowing visualization of length polymorphisms in different members of a species.

Mapping in the commercial strawberry is complicated by the octoploid genome. *F. vesca*, the diploid strawberry, has been used as a model for the octoploid strawberry. Davis and Yu (1997) generated a 445 cM long genetic linkage map consisting of seven linkage groups for *F. vesca*. Segregation data used for linkage analysis were obtained from the F₂ generation of a cross between 'Baron Solemacher' (BS), an alpine *F. vesca* variety, and WC6, a *F. vesca* clone collected from the wild in New Hampshire, USA. Segregation ratios were skewed, in five linkage groups, in all cases favouring the BS alleles over the WC6 alleles. The 80-marker map includes 64 dominant and 11 co-dominant RAPD markers, an alcohol dehydrogenase locus detected as a PCR-based sequence-tagged site, the phosphoglucose isomerase and shikimate dehydrogenase isozyme loci and the runnering and fruit-colour loci. The map contains a number of co-dominant RAPD markers, the detection of which was due in part to the use of template mixing methods for primer testing and marker analysis (Davis *et al.*, 1995).

Bulked segregant analysis has been used in strawberry to identify seven RAPD markers linked to the Rpf1 gene conferring resistance to *Phytophthora fragariae* var. *fragariae*, the causal agent of red stele root rot (Haymes *et al.*, 1997). The bulked DNAs represented subsets of an F₁ population obtained from the cross Md683 × 'Senga Sengana', which consisted of 60 plants and segregated in a 1:1 ratio for resistance or susceptibility to race 2.3.4 isolate NS2 of *P. fragariae*. Seven markers generated from four primers were linked to Rpf1. A linkage map of this resistance gene region was generated using JoinMap 2.0™ (Stam and Van Ooijen, 1995).

Based on this work, two dominant sequence-characterized amplified region (SCAR) markers have been constructed for gene Rpf1 (Haymes *et al.*, 2000).

2.3. Functional genomics

The enormous size of most plant genomes and the extraordinary abundance of repetitive sequences and allopolyploidy severely limit the number of plant species that will undergo complete sequencing. Genome investigations in strawberry will probably be through more limited sequencing and comparative genomics. Expressed sequence tags (ESTs) are seen as a quick way to gain sequence information of important genes (White *et al.*, 2000; Cordeiro *et al.*, 2001; Wang *et al.*, 2001). ESTs are short sequences obtained by analysis of cDNA clones. In strawberry, gene discovery by partial DNA sequence determination of cDNA clones could provide an effective means for building an information base for molecular investigations of mechanisms governing aspects of growth and development. Microarray experiments utilizing cDNA clones can be used to gain additional information about the potential roles of expressed genes at various stages of development (Minorsky, 2000; Mahalingam and Federoff, 2001). DNA microarrays coupled with the appropriate statistical analyses have been used in strawberry to study fruit flavour (Aharoni *et al.*, 2000). The formation of flavour compounds is closely correlated with metabolic changes during fruit maturation. A novel strawberry alcohol acyl-transferase (SAAT) gene that plays a crucial role in flavour biogenesis in ripening fruit has been identified. Although ESTs have yielded a vast amount of data, many genes will only be found by genome sequencing (Penn *et al.*, 2000).

Bacteria-based large-insert clones, including bacterial artificial chromosome (BAC), bacteriophage P1-derived artificial chromosome (PAC) and large-insert conventional plasmid-based clone (PBC), offer other potential means for accelerated sequencing of large, complex genomes (Zhang and Wu,

2001). They not only are capable of maintaining large DNA fragments of complex genomes (> 100 kb) in bacteria, but also are stable in host cells, have low levels of chimeric clones and facilitate DNA purification. A BAC library was constructed from high-molecular-weight DNA isolated from young leaves of papaya (*Carica papaya* L.) (Ming *et al.*, 2001). This BAC library contains 39,168 clones from two separate ligation reactions with an average insert size of 132 kb. Because of its relatively small genome (372 Mbp/1 C) and its ability to produce ripe fruit 9–15 months after planting, papaya offers a model plant species for studying genes that affect fruiting characters.

A physical map would permit the visualization of the position of sequence features such as genes and other markers on the chromosome. Accordingly, the genome is first digested into small pieces and maintained in a high-capacity cloning vector. To order genes and sequences on the chromosome, these fragments must be assembled into the linear order found on the chromosome. To aid assembly of the clones, a landmark map is created with markers dispersed throughout the map. Marker sequences that occur only once in the genome, known as sequence-tagged sites (STSs), are used. These STSs can be derived from ESTs, SSRs and single nucleotide polymorphisms (SNPs). In papaya a physical map based on BAC continuous sequences (contigs) to which ESTs have hybridized could be constructed. A physical map of the papaya genome should significantly enhance our capacity to clone and manipulate genes of economic importance in other fruit crops, by providing an understanding of the genome structure in terms of organization and content.

3. Micropropagation

Micropropagation of strawberry has been used for large-scale commercial propagation of elite selections and for analysis in a replicated trial of new releases. It also forms the basis for other *in vitro* techniques by providing a source of material and for main-

tenance of plants regenerated using these other techniques.

Strawberry plants are established *in vitro* from surface-sterilized axillary buds of glasshouse- or field-grown material. Buds are explanted on to semi-solid Murashige and Skoog (1962) (MS) medium containing $0.49 \mu\text{M}$ indolebutyric acid (IBA) and $8.9 \mu\text{M}$ benzyladenine (BA) and grown at 20°C under warm white fluorescent tubes at $70 \mu\text{mol}/\text{m}^2/\text{s}$ with a 16 h photoperiod. *In vitro* plants are propagated by subculture to fresh medium at 4–6-week intervals. Shoot elongation and rooting of *in vitro* plants are achieved on MS medium in the absence of growth regulators (Damiano, 1980; Graham *et al.*, 1995). Strawberry rooting *in vitro* and *ex vitro* has been compared. After a 4-week rooting period, plantlets that are rooted *ex vitro* have a larger root system. During subsequent growth, differences in development occur, e.g. more than twice as many runners are formed by *ex vitro*- than by *in vitro*-rooted plants (Borkowska, 2001).

In vitro-grown strawberry plants demonstrate changes in the large and small subunit of ribulose 1,5-biphosphate carboxylase (Rubisco), increased leaf chlorophyll concentration and C:N ratios (Premkumar *et al.*, 2001). Although micropropagated strawberries produce more flowers than conventionally propagated plants, they also produce more runners, which can lead to overcrowding and smaller fruit (Swartz *et al.*, 1981).

Micropropagation can be used to accelerate the breeding process by *in vitro* selection of genotypes for the trait(s) of interest. Rugienius and Stanys (2000) compared screening technologies of cold hardy strawberry seedlings and found a high correlation between cold hardiness *in vitro* and *in vivo* and between cold hardiness *in vitro* and winter hardiness *in situ*. Selection for salt tolerance has been evaluated by germinating seedlings *in vitro* on various concentrations of sodium chloride (Esensee *et al.*, 1991). *In vitro* testing of strawberry resistance to *V. dahliae* and *P. cactorum* has also been reported (Sowik *et al.*, 2001). *In vitro* flowering and fruiting of strawberry has been achieved by culture of shoot apices (Asao *et al.*, 1977).

4. Virus Elimination

Meristem tip culture alone or following thermotherapy has been utilized to eliminate viruses from infected material (Belkengren and Miller, 1962; Miller and Belkengren, 1963; McGrew, 1965, 1980; Vine, 1968; Nishi and Ohsawa, 1973). According to McGrew (1980), most strawberry viruses can be eliminated by meristem tip culture alone.

5. Somatic Cell Genetics

Strawberry breeding programmes have been hampered by several factors. The incorporation of exotic genes into existing germplasm to produce commercial cultivars through conventional breeding often involves many generations of back-crosses and is a lengthy process. Considerable time is required to produce recombinant plants from which accurate selection can be achieved. The highly heterozygous nature of the germplasm requires evaluation of large seedling populations and detection of infrequent recombinants is difficult. With conventional breeding, it is impossible to incorporate traits from non-related species and to achieve single gene changes without recombination. These obstacles result in high demands on field space, and require significant resources to be able to select, identify and evaluate desirable combinations. Biotechnology is therefore critical for the improvement of strawberry.

5.1. Regeneration

5.1.1. Somatic embryogenesis

Wang *et al.* (1984) reported somatic embryogenesis from cotyledons on MS medium supplemented with 22.6 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 2.2 μM BA and 500 mg/l casein hydrolysate. Maintenance of embryogenic cultures has been unsuccessful. Morphologically normal plants were obtained from the somatic embryos by alteration of growth regulator composition to include 0.29–1.44 μM gibberellic acid (GA_3)

and 0.54 μM naphthaleneacetic acid (NAA). Some effort would be required to further develop this technology for strawberry.

5.1.2. Organogenesis

Induction. There have been several reports of organogenesis from *Fragaria* spp. Liu and Sanford (1988) described efficient shoot organogenesis from leaf and runner tissues of 'Allstar' and 'Honeoye' on Linsmaier and Skoog (1965) (LS) medium. For 'Allstar', either 11.1 μM BA and 2.5 μM indolebutyric acid (IBA) or 13.3 μM BA and 0.5 μM IBA was optimum for induction, depending on the tissue source. Casein hydrolysate (400 or 600 mg/l) stimulated shoot production. 'Honeoye' produced shoots on LS medium containing 22.2 μM BA, 2.46 μM IBA and 400 mg/l casein hydrolysate. Shoots were induced from runner tissues of both cultivars on LS medium containing 44.4 μM BA, 11.4 μM indoleacetic acid (IAA) and 500 mg/l casein hydrolysate.

Shoot induction from leaf discs of 'Redcoat' has been described (Nehra *et al.*, 1989) on semi-solid medium consisting of MS salts and B5 (Gamborg *et al.*, 1968) vitamins supplemented with 10 μM each of BA and IAA. Low light intensity (12.5 $\mu\text{mol}/\text{m}^2/\text{s}$) greatly enhanced production. Shoot induction also occurred with nine other genotypes at varying frequencies, but with an intervening short callus phase. Shoot organogenesis was also achieved directly from leaf tissue with three cultivars on MS medium with BA and 2,4-D. Shoot induction, however, was infrequent, and varied between 15% and 35% for the three genotypes examined (Graham, 1990).

Shoot organogenesis has been induced from callus initiated from petiole or leaf lamina on semi-solid MS medium with BA and 2,4-D (Jones *et al.*, 1988). Calluses were formed from petioles of *in vitro* plants of 'Gorella' and 'Redgauntlet' on B5 medium with 2,4-D alone or in combination with either BA or kinetin. Shoots developed on calluses after 1–2 months; plant growth regulator content and genotype had marked effects on organogenesis. Organogenic callus has also been induced from the bases of

peduncles of excised flower buds of 'Fern', 'Hummi Gento', 'Gorella' and 'Redgauntlet' (Foucault and Letouze, 1987). Organogenic cultures have been induced from apical tissues of several cultivars on plant growth medium supplemented with BA or IBA, although high BA concentrations cause rapid shoot proliferation and axillary bud formation and can have a mutagenic effect (Kondakova *et al.*, 1983).

Maintenance. It has been possible to maintain organogenic suspension cultures of *F. vesca* for 2 years in induction medium formulation. Regenerants have been obtained from cell suspensions after the cell mass is decanted on to semi-solid regeneration medium (Infante *et al.*, 1996). Shoots derived from organogenic cultures can be micropropagated and rooted according to protocols described in Section 3.

5.1.3. Haploid recovery

Haploid strawberry plants would be useful for genetic and mutagenesis studies and for somatic hybridization. The production of polyhaploid plants ($2n = 4x = 28$) from octoploid strawberry ($2n = 8x = 56$) would be useful because of the heterozygosity of the species and the impossibility of fixing useful characters by selfing due to high polyploidy and inbreeding depression.

There have been several unsuccessful anther culture attempts involving strawberry (Rosati *et al.*, 1975; Laneri and Damiano, 1980; Niemirowicz-Szczytt *et al.*, 1983; Velchev and Milanov, 1984; Gavilova, 1985; Johansson *et al.*, 1987; Li *et al.*, 1988). All plants obtained from these studies were octoploid and must have developed either from somatic tissue of the anthers or as a result of cytological instability of the haplotetraploid callus derived from microspores. Polyhaploids have been produced by anther culture, followed by open pollination, selfing, crossing and back-crossing to *Potentilla* spp. (Niemirowicz-Szczytt *et al.*, 1983). Haploid production from strawberry has been achieved with 'Chandler', 'Honeoye' and 'Redchief' (Owen and Miller, 1996). Semi-solid MS medium

supplemented with 11.4 μM IAA, 4.44 μM BA and 0.2 M glucose is optimum for haploid shoot recovery. Another medium formulation containing IAA, BA and glucose, however, resulted in 19% shoot regeneration. Plants have been grown to flowering and fruit set. Chromosome counts of root tip cells have confirmed that the plants are haploid.

5.1.4. Protoplast isolation and culture

There have been few reports of protoplast isolation and culture involving strawberry. Leaf- and petiole-derived protoplasts (Johansson *et al.*, 1987; Nyman and Wallin, 1988, 1992) and protoplasts from calluses (Wallin, 1997) have been isolated. Protoplasts can be cultured, initially under continuous low light ($0.5 \mu\text{mol}/\text{m}^2/\text{s}$) on 8p medium (Glimelius *et al.*, 1986) with 0.4 M glucose, 4.5 μM 2,4-D and BA. After 21 days, the medium is replaced by modified 8p medium with 2% sucrose, 4.44 μM BA and 0.44 μM NAA. Shoot organogenesis is induced on MS medium containing 2% sucrose and 1.07 μM NAA with either 22.2 μM BA or 22.7 μM thidiazuron (TDZ). TDZ has been shown to enhance plant regeneration from protoplasts (Nyman and Wallin, 1992). Shoots can root on hormone-free medium. Measurement of the DNA content of these plants has revealed a range of ploidy levels.

5.2. Genetic manipulation

5.2.1. Mutation induction and somaclonal variation

Breeding objectives. Induced mutations, involving the alteration of one or a few specific traits of a commercially acceptable cultivar, could contribute to strawberry improvement. Nybom (1970) used gamma irradiation to generate mutants of strawberry; however, the mutant lines have not been useful in breeding programmes.

Accomplishments. Somaclonal variants of strawberry have been produced as a result of shoot tip and nodal culture and through *in*

vitro selection. Some concern has been expressed about the genetic stability of micropropagated plants. Genetic stability during micropropagation is controlled by numerous factors, including genotype, the presence of chimera tissue, explant origin, the *in vitro* protocol, medium composition and growth regulator concentration. Micropropagated strawberry plants, with sporadic occurrences of abnormal fruit setting and a hyper-flowering habit, have been observed in some cultivars, generally after several subcultures (Jemmali *et al.*, 1995; Boxus *et al.*, 2000). Hyper-flowering is unlikely to be a true mutation, but is possibly a result of DNA methylation. By decreasing the concentration of BA in the plant growth medium and limiting the number of subcultures, hyper-flowering can be eliminated.

Sansavini *et al.* (1989) reported a 5-year study of the most common somaclonal variants, i.e. white stripe, chlorosis and dwarfism. The frequency of somaclonal variation was found to vary between 0.048% and 0.461%, depending on the cultivar. In some cases, the variation was transient. Regenerants of five strawberry cultivars exhibited differences in callus and cell suspension growth rates and in isozyme patterns of acid phosphatase, peroxidase and glutamate dehydrogenase (Damiano *et al.*, 1995). Kumbhar *et al.* (1999) found little evidence for somaclonal variation in micropropagated strawberry (*Fragaria* × *ananassa* L.) grown on medium containing 5 and 15 µM BA or following cold storage. No mutations were observed for 246 loci amplified by the 29 random primers tested. Changes in methylation patterns of ribosomal DNA genes were observed in only one DNA sample from plants grown on medium with 15 µM BA and in one of the cold-stored plants.

Strawberry regenerants produced from anther culture have been demonstrated to vary with respect to earliness, calyx separation, rate of ripening and mildew (*Sphaerotheca macularis*) tolerance (Simon *et al.*, 1987). The ploidy level of plants from callus cultures of 'Bordurella' was doubled from 8x to 16x. These hexadecaploids had

very thick leaf blades and peduncles, and the soluble solid content of the fruits was significantly increased.

Somaclonal variants of strawberry with fungal resistance have been generated. Plantlets showing resistance to fungal wilt disease caused by *Fusarium oxysporum* f. *fragariae* (Toyoda *et al.*, 1991), to *P. cactorum* (Battistini and Rosati, 1991) and to *Alternaria alternata* have been reported. Cell lines resistant to *Colletotrichum acutatum* crude culture filtrates have also been selected, and regenerated plants showed polymorphism on the basis of RAPD analysis (Damiano *et al.*, 1997).

5.2.2. Somatic hybridization

Somatic hybridization offers the possibility of genetic exchange between the diploid *F. vesca* with the cultivated octoploid strawberry. Wallin (1996) regenerated plants from calluses in a fusion experiment between *Fragaria* × *ananassa* and *F. vesca* protoplasts. Protoplasts of *Fragaria* × *ananassa* resistant to hygromycin were fused to protoplasts of *Fragaria* × *ananassa* resistant to kanamycin. Plants that deviated morphologically from the parents were tested and found to have > 56 chromosomes. The increased ploidy level could have been caused by somaclonal variation or somatic hybridization.

5.2.3. Genetic transformation

Objectives. The targets for genetic modification have focused on traits of interest which are controlled by single genes, e.g. pest resistance, herbicide resistance, cold tolerance and ripening. For more precise modifications, however, an understanding of the regulatory pathways and the key genes controlling the desired characteristics is essential before genetic transformation can have a significant impact. For a fresh food product such as strawberry, the nature of the gene product must be carefully evaluated. In some cases targeting gene expression to the correct parts of the plant and away from the fruit would remove any risk, real or perceived, to the consumer.

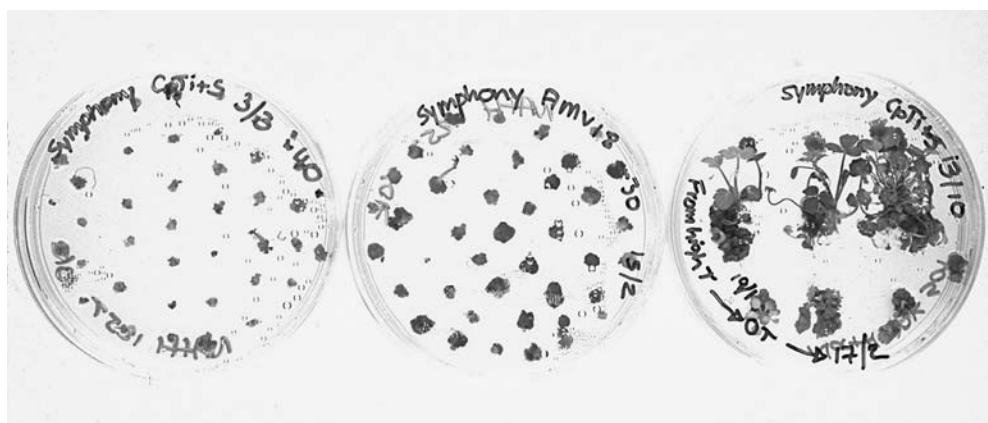


Fig. 18.1.1. *In vitro* selection for transformed strawberry.

Protocol. There have been several reports of *Agrobacterium*-mediated transformation (Graham, 1990; James *et al.*, 1990; Nehra *et al.*, 1990; Graham *et al.*, 1995; Mathews *et al.*, 1995, 1998; Martinelli *et al.*, 1997; Barcelo *et al.*, 1998). These studies have utilized *Agrobacterium* strains LBA4404 and EHA105 with pBin19 derivatives as the binary vector and a co-cultivation phase of a few days. Most reports involve transformation of a particular cultivar. Leaf- or stem-based organogenic regeneration systems, involving MS medium with BA together with 2,4-D or IAA, have been used. Selection of regenerants has occurred on medium with 25 mg/l kanamycin. Cefotaxime, carbenicillin and ticarcillin have been used to control *Agrobacterium* contamination after inoculation.

El Mansouri *et al.* (1996) and Haymes and Davis (1998) described *Agrobacterium*-mediated transformation of *F. vesca*. Leaves or shoot tissue were used as explants followed by regeneration on kanamycin medium. Improved transformation efficiency has been reported by combining *A. tumefaciens* infection with biolistic bombardment (de Mesa *et al.*, 2000).

Accomplishments. Various transgenic strawberries have been produced. Herbicide-tolerant strawberry plants expressing the phosphinothricin acetyl transferase gene,

which confers resistance to the herbicide glufosinate-ammonium, have been produced by Duplessis *et al.* (1995). Field trials indicated that four out of the 22 herbicide-resistant transgenic lines resembled the phenotypic characteristics of non-transformed control plants (Duplessis *et al.*, 1997).

Transgenic strawberry lines expressing the cowpea trypsin inhibitor (CpTi) have been evaluated under glasshouse (Graham *et al.*, 1997) and field (Graham *et al.*, 2002) conditions (Figs 18.1.1 and 18.1.2). Glasshouse bioassays carried out for vine weevil (*Otiorhynchus sulcatus*), a major strawberry pest, demonstrated a highly significant reduction in damage by weevil larvae in the transgenic lines. Field trials also demonstrated significant protection from vine weevil damage (Graham *et al.*, 2002) with no significant impact on non-target pests. For a review of potentially useful anti-nutritional genes with reference to strawberry, see Watt *et al.* (1999). Transformation of strawberry with the lectin *Galanthus nivalis* agglutinin (GNA) has been carried out alone and in combination with CpTi (K. Watt, personal communication). Bioassays involving the GNA plants failed to demonstrate any reduction in vine weevil attacks (J. Graham, unpublished data) compared to non-transgenic controls.

Transgenic plants have been recovered that contain the gene *S*-adenosylmethionine

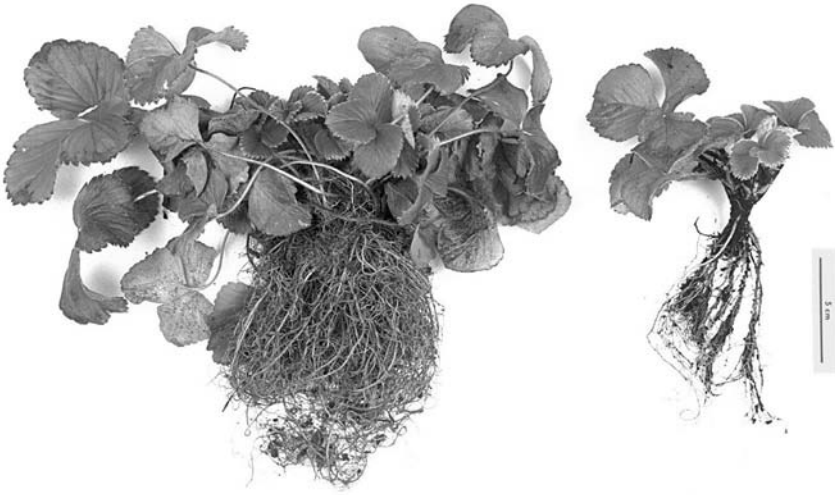


Fig. 18.1.2. Transgenic strawberry containing the CpTi gene after vine weevil feeding.



Fig. 18.1.3. Genetically transformed strawberry under field conditions.

hydrolase (SAMase) (Mathews *et al.*, 1995) for control of ethylene biosynthesis. Although strawberries are classed as non-climacteric fruit, they do respond to low levels of ethylene. Any reduction in ethylene biosynthesis, especially postharvest, could slow down the softening process. Another

strategy aimed at the control of fruit softening, and involved transformation with an antisense sequence of the strawberry pectate lyase gene. Transgenic lines were significantly firmer than control lines exhibiting a 30% decrease in pectate lyase gene expression (Jimenez-Bermudez *et al.*, 2002).

To improve cold tolerance, transgenic strawberries have been developed that express anti-freeze proteins isolated from winter flounder (Wongruong, 2000). This gene delays the formation of ice crystals by binding anti-freeze proteins with water molecules. Transgenic strawberries exhibited a significant reduction in cell damage and electrolyte leakage. Two transgenic lines of 'Honeoye' have been developed which express CBF1, a cold-induced transcription factor (Owens *et al.*, 2002a), at a low level. Freezing tolerance values in these lines were significantly greater than for the control.

5.3. Cryopreservation

The effects of pre-freezing treatments, cryoprotectants and cooling rate on survival of strawberry meristems have been studied (Kartha *et al.*, 1980). A cryopreservation protocol has also been established for strawberry suspension cultures (Yongjie *et al.*, 1997). Cells are collected at the beginning of their exponential growth phase, pre-treated at 0°C for 60 to 180 min in plant vitrification solution 2 (PVS2) (Sakai *et al.*, 1991), consisting of glycerol (30%), ethylene glycol (15%) and dimethyl sulphoxide (DMSO) (15%), cooled at 0.5°C/min to temperatures ranging between -35 and -45°C, and immersed rapidly in liquid nitrogen (-196°C). Thawing the cryopreserved cells in a water bath at 20 or 30°C is essential for cell viability.

6. Conclusions

Strawberry improvement will increasingly rely on the application of biotechnology to develop improved cultivars suited to the changing needs of growers and consumers. Previously difficult or impossible parental combinations as well as the integration of genes from other genera, manipulation of complex pathways and a greatly increased understanding of the germplasm and of the genome are feasible through application of these new techniques. Great and generally unexplored potential exists in the application of mutation breeding, perhaps as an alternative to genetic transformation in the short term in Europe. Somaclonal variation may prove to be a bonus in the application of other micropropagation techniques. New marker techniques such as SSRs and SNPs when developed and applied to strawberry should be useful for developing saturated linkage maps for mapping quantitative trait loci (QTLs).

Progress in strawberry is some way behind that in other major commodity crops in the application of these techniques. This, however, is due to the cost of developing and applying these technologies to strawberry, where limited funding exists. The recalcitrant nature of strawberry crops in terms of cell culture techniques and also ploidy complications for mapping have also delayed progress.

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18.2 *Malus* × *domestica* Apple

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1. Introduction

1.1. Botany and history

Apple is in the genus *Malus* Miller in the subfamily *Maloideae*, one of four subfamilies in the family *Rosaceae*. *Malus* Mill. is comprised of small, much-branched deciduous trees that are grown in orchards or as ornamentals. *Malus* is said to contain from eight to 78 species, but 25 species is often a consensus figure (Phipps *et al.*, 1990). Classification as to species is difficult due to the great genetic diversity, the hybridization potential and the presence of apomixis and polyploidy (Campbell *et al.*, 1991). Different classifications have been based upon morphological traits, chromosome number, crossability or molecular analyses. The presence of a deciduous or persistent calyx is often used as a criterion. The taxonomic classification of *Malus* and a brief history were reviewed by Luby (2003).

Malus is a pome fruit, i.e. a fleshy accessory fruit consisting of a central core with seeds enclosed by a papery capsule of fused carpels. The outer thickened fleshy layer is from the enlarged receptacle and is the edible part of the fruit. Fruit of *Malus* lack the stone or grit cells found in *Pyrus*, another pome fruit. *Malus* is also distinguished from *Pyrus* by its soft leaf surfaces, acutely rather than callously serrate leaf margins and broad pubescent or tomentose

winter buds. Flower clusters are mostly simple without a columnar central stalk. The hypanthium is open in anthesis and not closed about the styles. Styles are more or less connate basally (Bailey, 1975). Apple flowers are white to pink or carmine, in cymes. The petals are usually suborbicular or obovate. The stamens range from 15 to 50 with usually yellow anthers. The ovary is inferior and the two to five styles are connate at the base.

Malus is characterized by great phenotypic and genetic diversity. High heterozygosity is reinforced by self-incompatibility and inbreeding depression. The 'cultivated' apple is a catch-all term that encompasses any cultivated type. It is a complex interspecific hybrid. While the binomial *Malus* × *domestica* Borkh. has become the standard designation (Korban and Skirvin, 1984), *M. pumila* Mill. continues to be used.

The origin of apple continues to be the subject of investigation. Vavilov (1926) suggested that the wild apple of Turkestan and its relatives were the progenitors of domesticated apple, based upon similarities, and this hypothesis has been supported by analysis of nuclear ribosomal DNA (rDNA) and chloroplast DNA (cpDNA) (Harris *et al.*, 2002). *M. sieversii* was important in the origin of the cultivated apple and it is likely that it hybridized with *M. prunifolia*, *M. baccata* and *M. sieboldii* as material was disseminated to the east. Hybridization with *M. turkmenorum*

and *M. sylvestris* was likely as it progressed west (Juniper *et al.*, 1999).

Apple germplasm and the maintenance of genetic diversity is important to future breeding and biotechnology improvements. Apple germplasm was reviewed by Way *et al.* (1990). Zhou (1999) reviewed apple resources in China, with an emphasis on wild species, their geographic distribution, characteristics and use, and Dzhangaliev (2003) reviewed the wild apples of Kazakhstan. Genetic variation in wild apple (*M. sylvestris*) in Belgium was examined using amplified fragment length polymorphisms (AFLPs) and simple sequence repeat (SSR) markers and, while cultivated genotypes were present in the wild, gene flow between wild and cultivated gene pools was almost absent (Coart *et al.*, 2003). In many countries the collection, conservation, evaluation and documentation of *Malus* germplasm is a priority (Forsline, 2000). Information on *Malus* in the USA can be found at <http://www.ars-grin.gov> as part of the Germplasm Resources Information Network (GRIN).

The apples that are native to North America bear no resemblance to cultivated apple. Only four *Malus* species are endemic to North America: *M. fusca* (Raf.) C.K. Schneid. (Oregon crab), *M. coronaria* L. (sweet crab apple), *M. angustifolia* (Aiton.) Michx. (southern crab) and *M. ioensis* Wood (Brit.) (prairie crab). There has been little natural hybridization with cultivated apple. The phosphoglucosomerase 3 (PGM-3) isozyme that is characteristic of *M. × domestica* was found in only one of 72 natural populations of North American *Malus* studied (Dickson *et al.*, 1991).

Apples have been cultivated since at least 3000–4000 BC and they have been featured prominently in literature, poetry, art and folklore since that time. The history of the apple is very rich and many books feature a review of its history in a particular region or time period or for a particular use. Apples have been symbolic since early time and were often featured in mythology. Interesting discussions of the history of apples and apple culture are found in Morgan and Richards (1993), Browning

(1998) and Pollan (2001), among other sources.

1.2. Importance

Domesticated apple is one of the most important fruit crops worldwide. According to FAOSTAT (2004), world apple production exceeded 57,000,000 t in 2003. Leading producing nations include (in descending order) China, USA, France, Poland, Turkey, Italy and Russia. Apple production is exceeded by *Musa* (banana and plantain), citrus and grapes. While there are well over 6000 documented apple cultivars, commercial production still relies on fewer than 20 major cultivars, with 'Delicious', 'Golden Delicious', 'Granny Smith', 'Fuji' and 'Gala' accounting for 61% of the total production (O'Rourke, 2003).

Apples are processed into many products including juice, apple sauce, slices (dried, frozen and canned) and cider (sweet and hard) (Downing, 1989). Many *Malus* species are also important in the landscape market and in breeding for better ornamental types (Fiala, 1994). Markets in the future may emphasize the nutritional aspects of apples, such as increased antioxidant level. Non-browning genotypes would be useful for fresh-cut products.

1.3. Breeding and genetics

Apple has a haploid chromosome number of $x = 17$, and triploids, tetraploids and hexaploids have been documented. Although apple is believed to be an ancient allopolyploid it behaves as a functional diploid in breeding. Breeding of apples has been reviewed by Brown (1975), Brown and Maloney (2003), Janick *et al.* (1996) and Laurens (1999). Other reviews have emphasized a particular area of breeding, e.g. scab resistance (Crosby *et al.*, 1992; Bus *et al.*, 2000), or by specific locations, e.g. Soejima *et al.* (2000). Interspecific hybridizations have been used in breeding apple to improve traits, e.g. cold hardiness, disease resistance and other traits (Korban, 1986). Reviews of

genetics or qualitative genes used in breeding include Brown (1992) and Alston *et al.* (2000).

1.3.1. Rootstocks

Major breeding objectives. Apple rootstocks, their characteristics, their problems, types of rootstocks available, breeding programmes, objectives and outcomes have been reviewed by Ferree and Carlson (1987), Rom (1987), Wertheim (1998) and Webster and Wertheim (2003). Rootstocks help to control scion vigour and cropping, and influence the scion's response to biotic and abiotic stresses. Major breeding objectives in the development of apple rootstocks include tree size control (dwarfing), tolerance of cold (hardiness), tolerance of pathogens and pests and tolerance of wet or dry soil conditions. Webster and Wertheim listed attributes of the 'ideal' rootstock, which included long-term graft compatibility and good health, e.g. freedom from virus and from bacterial diseases caused by *Agrobacterium* or *Erwinia*. Important to the nursery trade are ease of propagation and good nursery performance. Dwarfing, ability to induce precocious and consistent cropping, resistance/tolerance of biotic and abiotic stress and freedom from suckers and burr knots are essential for growers. Good anchorage is also important.

Specific disease and pest resistances being targeted in breeding include resistance to crown and root rot (*Phytophthora* spp., especially *P. cactorum*), resistance to woolly apple aphid (WAA) (*Erisoma lanigerum* Hausmann) and resistance to fire blight (*Erwinia amylovora* Burrill Winslow *et al.*). Individual programmes often have additional resistance goals that are related to regional problems, e.g. *Nectria* canker resistance in Europe.

Breeding accomplishments. In 1917, East Malling Research Station (UK) established the first rootstock breeding programme. In the 1920s they collaborated with the John Innes Institute (UK) to develop a series of rootstocks with resistance to WAA derived from 'Northern Spy'. The stocks also had other desirable characteristics. The first

series was called 'Merton Immune' and this was followed by a 'Malling Merton' series, which would become among the best known and most widely grown rootstocks to date. Extensive descriptions of the Malling rootstocks are found in Ferree and Carlson (1987), Wertheim (2003) and Webster and Wertheim (1998).

The breeding programme at Cornell University's New York State Agricultural Experiment Station (Geneva, NY) was initiated in 1953 with 158 openly pollinated seedlings of the very dwarfing 'M.8' (Johnson, 1999). Many of these selections were discarded due to problems with suckering. Later, more specific goals included use of *Malus* × *robusta* (Carr.) Rehd. cv. 5 ('Robusta 5') for its resistance to fire blight and WAA and *Malus* × *atrosanguinea* (Spaeth) Schneid., *M. fusca* and *Malus* × *sublobata* 'Novole' for resistance to fire blight (Cummins and Aldwinckle, 1983). 'Novole' was released in 1982 as a full vigour rootstock that is relatively unpalatable to voles and also has resistance to fire blight, *Phytophthora* and tomato ring spot virus. Selections of the CG (Cornell Geneva) series are designated as 'Geneva'TM or G series.

The fire blight resistance of many of the CG stocks is excellent. Norelli *et al.* (2003a) examined resistance to *E. amylovora* and found that 'Budagovsky 9', 'Ottawa 3', 'Malling 9' and 'Malling 26' were the most susceptible. 'Geneva 11', 'Geneva 5', 'Geneva 16', 'Geneva 30', 'Pillnitzer Au51-11', 'Malling 7' and several breeding selections were the most resistant.

Robinson *et al.* (2003a) discussed the performance, disease resistance and commercialization of the CG/Geneva series of rootstocks. These stocks were included in the USA national rootstock testing trial, the NC140. In 1992, 18 CG/Geneva stocks and five Malling rootstock controls, with 'Liberty' as a scion cultivar, were established in a multi-site (13 locations) replicated trial. In 1993, 23 CG/Geneva stocks and four Malling rootstock controls were also established as part of the NC140 trials (Robinson *et al.*, 2003b).

In 1959, Michigan State University planted open-pollinated seed from Malling

rootstocks ('M.1'–'M.16'), 'Alnarp 2' and 'Robusta 5' and selected clones with desirable nursery characteristics and freedom from WAA. Fifty-six selections were propagated and 'Michigan Apple Clone 9' ('MAC 9') was named 'Mark' in 1979 as an 'M.9' equivalent with less suckering. Unfortunately, 'Mark' had other problems that restricted its commercialization.

Breeding objectives of the rootstock programme in Quebec, Canada, include hardiness, yield efficiency, precocity, dwarfing ability and ease of propagation. Crosses include 'Robusta 5' $\times$ 'M.27', 'Robusta 5' $\times$ 'Budagovsky 57490' and open-pollinated 'Ottawa 3' (Khanizadeh *et al.*, 2000).

Selections remain from the breeding programme of the Horticulture Research Institute of Vineland, Ontario, Canada. These originated from open-pollinated seed of the crabapple 'Kerr', with 'M.9' the likely pollen parent. Results from trials in North America suggest that the V selections are winter hardy and have low susceptibility to fire blight. Several of the V stocks are prone to suckering.

A series of rootstocks developed by V.I. Budagovsky at the Michurinsk College of Agriculture in Russia bear his name. These selections are also known as the Bud. series or B series, of which 'B.9' is the best known (Wertheim, 1998).

In Japan the moderately vigorous rootstocks 'Marubakaido' (*M. prunifolia* var. Ringo) and 'Mitsubakaido' (*M. sieboldii*) are used, but they are not suitable for high density due to their size and susceptibility to virus. In 1972, crosses were made with 'Marubakaido' $\times$ 'M.9'. 'Marubakaido' was selected as a parent for its resistance to WAA and to *Phytophthora* rot, and 'M.9' was selected for its dwarfing character. Ten clones were selected that have good resistance to crown rot, propagate from hardwood cuttings, and (except for JM 2) appear to have resistance to WAA (Wertheim, 1998). Details about the breeding programme and performance of the JM stocks is detailed in Bessho and Soejima (1992). Oraguzie *et al.* (2003) also reviewed breeding of apple in Japan. Research to produce apomictic rootstocks is another area of interest (Kon *et al.*, 2000).

Dresden-Pillnitz in Germany started breeding apple rootstocks in 1911, with dwarfing, good anchorage, early and high productivity and resistance to scab, mildew and WAA as key objectives (Wertheim, 1998). A cross of 'M.9' $\times$ 'M.4' resulted in numerous selections, and one clone from these selection was named 'Pillnitzer Supporter®4' (Fischer, 1999). Wertheim (1998) and Fischer (1999) provide additional information on the Pillnitz-Supporter clones nos 1, 2 and 3.

Rootstocks from the breeding programme at Balsgard, Sweden, are designated 'BM' for Balsgard *Malus*. Winter hardiness was a key objective. In 1974 'Bemali' was named from this programme. Although less vigorous and more efficient than 'M.7', its low productivity relative to 'M.9' led to the conclusion that it is not suited to high-density systems (Wertheim, 1998).

Breeding of rootstocks in Poland was reviewed by Jakubowski and Zagaja (2000). This programme stressed hardiness since the Malling rootstocks were not sufficiently hardy. Malling stocks were crossed with 'Antonovka'. Selections are designated by the letter 'P' followed by a number, e.g. P1. Many clones are being tested in trials, with four clones obtained from a mutation-breeding project using γ -irradiation. Some of the P clones are susceptible to WAA and fire blight.

Two selections, 'Voinesti 1' and 'Voinesti 2', have been released by the research institute in Voinesti, Romania. There are also two breeding programmes in the Czech Republic. These stocks are designated JTE or JOH followed by a letter, such as JOH-A. Four JTE selections have received plant variety rights in the Netherlands.

Genetic studies of dwarfing and other traits of interest in rootstock breeding have accelerated. Sandanayaka *et al.* (2003) examined characteristics associated with resistance to WAA in three rootstocks. Molecular markers promise to facilitate rootstock improvement, especially with respect to traits associated with dwarfing (Gardiner *et al.*, 2003). Transgenic approaches to apple rootstock improvement also hold promise for incorporating resistance and for improving propagation.

1.3.2. Scions

Major breeding objectives. Major breeding objectives were reviewed by Janick *et al.* (1996), Laurens (1999) and Brown and Maloney (2003). Information on specific objectives, e.g. mutation breeding, increasing yield and breeding for resistance to environmental stress, was reviewed in Janick and Moore (1983). Apple breeding programmes by country were outlined in Brown and Maloney (2003).

When Laurens (1999) surveyed 42 breeders in 29 countries about their breeding objectives, a common goal was the development of new cultivars with high fruit quality coupled with disease or pest resistance. Resistance to apple scab (*Venturia inaequalis* (Cooke) G. Wint.) and to powdery mildew (*Podosphaera leucotricha* (Ell. & Ev.) Salm.) were a priority of many programmes. Development of tree habit for high productivity and annual bearing was also a common objective. Adaptation to extreme climates was the goal of several programmes.

Breeding low-allergenic cultivars has become of interest, especially in Europe where allergic reactions to apples are more severe. Son *et al.* (1999) found that the differences in allergenicity among cultivars were related to different expression levels of the allergens. Allergenicity of new releases must not be elevated.

Cultivars developed for the processing markets need to have specific attributes depending on the product: cider (hard and sweet), apple sauce, slices and 'fresh-cut' (Crassweller and Green, 2003). Apples with natural resistance to flesh browning after cutting are desirable for processing and are being developed.

We must ensure that new cultivars that are developed are an improvement over those currently available. New cultivars must be different, distinctive, better, more nutritious, disease resistant or of superior quality. Development of apples with a unique appearance and/or flavour is needed to enhance marketing opportunities. Examples of unique attributes include red flesh, full surface russet, unusual shapes,

colours, flavours or enhanced quality. These new distinctive cultivars must have consistent quality across production sites and must be annually productive for a consistent supply.

Breeding ornamental *Malus* (crab apples) for the home market or landscape trade must stress disease resistance. There are a wide array of plant and leaf forms, flower types and disease resistances in *Malus* species that can be used to breed improved ornamentals but will also add to our knowledge of these genes for use in scion cultivars.

Characterization and use of diverse germplasm is a goal of many breeding efforts. There is reduced genetic diversity in apple from the use of a restricted number of parents in breeding. Examining resistance genes of material collected from the centre of origin is also an objective.

Breeding accomplishments. New apple cultivars continue to be released by programmes worldwide (Janick *et al.*, 1996; Laurens, 1999), with descriptions published in trade magazines and in scientific literature. New cultivars can be very profitable and marketing clubs are being formed to offer exclusive access to some new cultivars. In developing improved cultivars, we benefit from multi-disciplinary studies. Advances in pathology, entomology, genetics and food science provide new tools and understanding of traits. Sequences from well-characterized plant genomes such as *Arabidopsis* are providing the opportunity to screen for homologous genes in apple.

For disease resistance breeding, molecular markers, better understanding of the pathogen and its interactions with the host and better screening techniques are allowing us to breed more durable resistance. Progress in incorporating resistance to apple scab and powdery mildew is evident in Section 2.2 on molecular markers, mapping and cloning. Resistance sources have been identified and used and many new resistant cultivars have been released. Breeding for resistance to *Alternaria alternata* is ongoing in Japan (Oraguzie *et al.*, 2003) and in Korea, and efforts to impart resistance to *Valsa* canker and to apple canker (*Nectria galligena*) are

also under way. Norelli *et al.* (2003b) reviewed new technologies to enhance host resistance to fire blight (*E. amylovora*) in apple. Unfortunately, while multiple disease-resistant lines have been developed, many have lacked the commercial quality needed to be competitive. However, organic apple production is being realized and new cultivars have been identified that have potential for this system (Weibel and Haseli, 2003).

The development of insect-resistant apple cultivars and rootstocks has progressed at a slower rate than with disease resistance, but advances are being made. Three genes for resistance to WAA (*E. lanigerum* Hausm.) have been identified and studied. The resistance of 'Northern Spy' (*Er1*), 'Robusta 5' (*Er2*) and 'Aotea' (*Er3*) was evaluated by Sandanayaka *et al.* (2003), who concluded that WAA in New Zealand may have overcome the *Er3* resistance. They suggested strategies for pyramiding to provide durable resistance.

Our understanding of the resistance to rosy leaf-curling aphid (*Dysaphis devector* Wlk.) has progressed from identifying sources of resistance, studying the inheritance of resistance and biotypes of the aphid, developing markers (Roche *et al.*, 1997a,b) and fine mapping (Cevik and King, 2002a) to determining the location of the *Sd-1* locus on a bacterial artificial chromosome (BAC) library contiguous sequence (contig) (Cevik and King, 2002b).

Sherman and Beckman (2003) reviewed breeding for climatic adaptation in fruit crops and cited different factors and mechanisms for adaptation. Adaptation to cold climates is a goal of many programmes, especially those in countries at the extremes of apple culture. Breeding for adaptation to low-chill regions is also an important goal of several programmes. Breeders are investigating the inheritance of factors associated with this complex trait, looking for molecular markers and using more diverse germplasm for low-chill breeding (Labuschagne *et al.*, 2002). Labuschagne *et al.* (2003) found a significant response to selection for budbreak number (NB) based on data recorded on 1-year-old shoots of apple seedlings and branches from mature seedling trees. They

suggested that combining selection using genetic variation between crosses and within crosses was the best way to increase the frequency of seedlings with increased budbreak and improve adaptation to low winter chilling environments.

Laurens (1999) found that genetic improvement of plant form is a major goal of many programmes, especially for breeding spur type, columnar (reduced branching) and tip-bearing types. This work will be enhanced by markers developed or being developed for these traits. Lauri *et al.* (1997) raised important questions on the role of morphological traits in selection of non-biennial bearing. Research on how different genes for plant form interact, which are dominant and the extent of environmental effects is under way (Fideghelli *et al.*, 2003). Currently, there are markers for columnar or reduced branching habit, weeping habit, spur habit, tip-bearing and compact types (Tartarini and Sansavini, 2003). Characterization of the diverse plant forms in apple will add to our understanding of the genetics of plant architecture.

Our understanding of self-incompatibility (*S*) alleles has increased markedly (Broothaerts and Van Nerum, 2003) and this knowledge can be used in the design of controlled hybridizations. Studies of partially self-compatible cultivars such as 'Megumi' will also aid in the development of self-fertile cultivars. Mutation breeding will also be used to see if self-fertile mutants can be obtained. Genetic improvement for self-fertility will use classical breeding and transgenic approaches (Van Nerum *et al.*, 2001), including *S* gene silencing (Broothaerts *et al.*, 2003).

Alston *et al.* (2000) summarized other traits under relatively simple genetic control that could be investigated. In *Malus*, there are genes for dwarfing, pale green lethal, yellow leaf mottle, red pigmentation, partial and full fruit russeting, double flowering and other resistances to diseases and pests that are amenable to study. Knowing what cultivars are heterozygous for pale green lethal and for dwarfing genes, which are common in scab-resistant material, would aid parental selection to avoid generating the

25% homozygous recessive progeny that are not useful in cultivar development.

Genetic investigations are being aided by large cooperative projects, e.g. the Durable Apple Resistance in Europe (DARE) programme, with its emphasis on clonal propagation and multi-site research. Studies using clonal propagation across sites provide valuable information on environmental effects and pathogen differences for mapping and genetic studies. The potential value of haploids in apple was reviewed by Lespinasse *et al.* (1999). Doubled haploids exist in apple (Hofer *et al.*, 2002) and will be used to study the inheritance of key traits.

Statisticians and geneticists are becoming interested in apple genetics. Durel *et al.* (1998) used pedigree information to estimate genetic parameters from large unbalanced data sets in apple. Genetic parameters in a recurrent selection programme in apple were estimated by Oraguzie *et al.* (2001). Quantitative trait locus (QTL) studies in apple include those on growth and development of seedling apple trees (Conner *et al.*, 1998), studies of fruit texture and quality attributes (King *et al.*, 2000, 2001) and examination of physiological traits (Liebhard *et al.*, 2003a). A better understanding of complex traits is needed because these traits currently are not amenable to genetic engineering.

Quantification of quality attributes is becoming a high priority in many breeding programmes, especially for flavour and for textural components of firmness, crispness and juiciness. Instruments used to characterize quality attributes are also being evaluated and contrasted with results from sensory testing. Sensory interpretation of instrumental measurements of fruit texture and the sweet and acid taste of apple fruit was conducted by Harker *et al.* (1996, 2002a,b). The DARE programme now emphasizes sensory testing and consumer perceptions and expectations. Breeders are incorporating sensory testing into their programmes. Hampson *et al.* (2000) used sensory evaluation as a selection tool in apple breeding. Spider plots allow advanced selections to be compared with commercial standards. Deslauriers *et al.* (1999) discussed the use of descriptive sensory analysis and cor-

respondence analysis to select apples for fresh and processing markets, while Cliff *et al.* (1999) evaluated hedonic scores and R indices for visual, flavour and texture preferences of apple cultivars by Canadian consumers. These studies are helping to define methods for measurement of quality and to study the genetics of these important quality traits.

New cultivars are being developed that have higher levels of vitamin C (ascorbic acid). However, vitamin C contributes about 12.8% to the total antioxidant activity of apples (Eberhardt *et al.*, 2000; Lee, K.W. *et al.*, 2003). The health benefits and antioxidant activities of apples, the types of phenolics, their stability, the tests used to quantify them and cultivar differences are being examined (Treutter, 2001; Schirrmacher and Schempp, 2003; Schmitz-Eiberger *et al.*, 2003). Understanding the complexity of antioxidants, their interactions and their role in fruit coloration, maturation, storage and pathogen defence will continue to be of interest in conventional breeding and biotechnology.

More detailed genetic studies of post-harvest quality and susceptibility to disorders are being conducted (Kochar *et al.*, 2003). Apple fruit susceptibility to storage disorders was studied in 30 half-sib families by Volz *et al.* (2001). Soft scald was the only disorder that was highly heritable across the two sites in the study. Internal and external bitter pit and brown heart had strong site × family interactions and were moderately heritable at each site, but not across the two sites. Extensive use of the soft scald- and bitter pit-susceptible cultivar 'Honeycrisp' as a parent may also add to our understanding of the inheritance of these disorders.

Great progress is being made in our understanding of both qualitative and quantitative traits important in the genetic improvement of apples. Molecular markers allow us to genotype seedlings at an early stage. Such pre-selection is especially important in apple, where a long juvenile period and large tree size add to the cost of maintaining large seedling populations. The new techniques being developed, coupled with a better understanding of genes of interest,

will allow us to make vast improvements in apple cultivars of the future.

2. Molecular Genetics

2.1. Gene cloning

Cloning genes from apple has accelerated greatly. Initial research focused on either differential gene expression or looking for homologues of well-characterized genes. Research on the factors affecting fruit ripening and softening was of interest to several groups, who researched the ripening-related aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase.

Dong *et al.* (1991) cloned a complementary DNA (cDNA) encoding ACC synthase and studied expression in ripening apple fruit. A cDNA coding for an ACC oxidase homologue from apple fruit was then sequenced (Dong *et al.*, 1992). Molecular studies of ACC synthase and ACC oxidase suggested that at least two allelic forms of ACC oxidase exist (Castiglione *et al.*, 1999). An allele of ACC synthase (Md-ACS1) was determined to account for low levels of ethylene in some apple cultivars (Harada *et al.*, 2000). Dong *et al.* (1998a) monitored expression of ACC oxidase and polygalacturonase (PG) in three apple cultivars, while Atkinson *et al.* (1998) looked at ripening-specific gene expression of ACC oxidase and PG and promoter analyses in tomato. Yao *et al.* (1999) found that a gene encoding PG inhibitor in apple fruit is developmentally regulated and activated by wounding and/or fungal infection. When PG was overexpressed in transgenic apples, novel phenotypes were observed, reflecting changes in cell adhesion (Atkinson *et al.*, 2002).

Other research included isolating and characterizing genes differentially expressed early in apple fruit development (Dong *et al.*, 1997), studying expression of cDNA from apple encoding a homologue of DAD1, an inhibitor of programmed cell death. Apple was found to contain at least two homologues of DAD1 and these were induced by pollination and by flower and leaf senescence (Dong *et al.*, 1998b). Knotted1-like

homoeobox genes were expressed during growth and development (Watillon *et al.*, 1997), while MDH1, an apple homoeobox gene belonging to the BEL1 family, was found to be involved in control of plant fertility. Transgenic plants of *Arabidopsis* with MDH1 were dwarf (Dong *et al.*, 2000). Sakamoto *et al.* (1998) determined that at least two different types of homoeobox genes exist in apple.

Understanding self- and cross-incompatibility in apple is an important issue for ensuring pollination and in controlled hybridizations. Sassa *et al.* (1994, 1996) first used molecular approaches to study apple incompatibility. Broothaerts *et al.* (1995) cloned two *S* alleles and helped in the development of a polymerase chain reaction (PCR)-based identification system (detailed in section 2.2.). Partial genomic sequences of the *S*2 and *S*9 alleles were obtained from the self-compatible cultivar 'Megumi'. In 2002, both the *S*25 and *S*10 cDNAs from 'McIntosh' apple were cloned and identification methods were reported (Kitahara and Matsumoto, 2002a,b).

Anthocyanin biosynthesis genes preferentially expressed in apple fruit skin were cloned and analysed by Kim, S.-H. *et al.* (2003). The cDNAs encoding flavanoid-3-hydroxylase (FSH), dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS) and uridine diphosphate (UDP)-glucose : flavonoid 3-O-glucotransferase (UGT) were found to have high homology to sequences from other plants. The mRNAs of these genes were detected preferentially in the apple skin tissue and were induced by light. Transcripts were abundant in red-skinned cultivars but rare in non-red fruit, suggesting their involvement in apple skin colour.

Cloning of genes involved in disease resistance has also been a priority in research. Oh *et al.* (2000) isolated a cDNA encoding a 31 kDa, pathogenesis-related 5/thaumatin-like (PR5/TL) protein abundantly expressed in apple fruit of 'Fuji'. Poupard *et al.* (2003) determined that a wound- and ethephon-inducible PR-10 gene subclass from apple was expressed differentially during infection with a compatible and an incompatible race of *V. inaequalis*. The

extensive research leading to the cloning of the V_f scab resistance gene is detailed in Section 2.2.

Sanchez-Torres and Gonzalez-Candelas (2003) isolated and characterized genes differentially expressed during the interaction between apple fruit and *Penicillium expansum*. They identified among the differentially expressed genes one fungal gene encoding an unknown protein and two apple genes homologous to a β -glucosidase and a phosphatase 2C, respectively. These genes were expressed exclusively during the infection process.

Research on apple allergens has raised questions about allergenicity and disease resistance. Mal d1 protein, a major apple allergen, is classified as a pathogenesis-related protein 10. When a promoter of the apple Ypr10 gene was isolated and characterized, it was found to be both stress- and pathogen-inducible (Puhlinger *et al.*, 2000). Diaz-Perales *et al.* (2002) reported on cDNA cloning and heterologous expression of the major allergens from peach and apple belonging to the lipid-transfer protein family. Krebitz *et al.* (2003) examined plant-based heterologous expression of the Mal d 2 apple allergen, a thaumatin-like protein, and characterized it as an anti-fungal protein.

To understand the postharvest disorder superficial scald, researchers have started to clone and characterize gene products that regulate production of α -farnesene in apple peel since α -farnesene is thought to be a precursor of scald. The initial rate-limiting enzyme in the pathway towards scald is 3-hydroxy-3-methylglutaryl-coenzyme A (CoA) reductase (hmg). Rupasinghe *et al.* (2001) cloned *hmg1* and *hmg2* cDNAs and examined their expression and activity in relation to α -farnesene synthesis in apple. Pechous and Whitaker (2002) also cloned *hmg1* and examined its bacterial expression.

Apple polyphenol oxidase (PPO) has been a focus of research for several groups, with Boss *et al.* (1995) finding apple PPO cDNA to be up-regulated in wounded tissues. Kim *et al.* (2001) used expressed sequence tags (ESTs) from 'Fuji' to identify

two partial PPO clones, and determined that they were differentially expressed during vegetative and reproductive development and in response to wounding. Transgenic lines with antisense PPO had lower browning than control shoots (Murata *et al.*, 2000). Broothaerts *et al.* (2000b) developed a fast leaf PPO assay for screening transgenic plants of apple and tobacco.

Research on plant hormones is also of interest. The gibberellin 20-oxidase gene was researched in apple by Kusaba *et al.* (2000). Southern blot analysis revealed that at least two homologous genes exist in apple. The gene consists of three exons and two introns. Gene expression in immature seeds was activated by rapid growth of the fruit. Wegrzyn *et al.* (2000) identified a novel α -amylase gene transiently up-regulated during low-temperature exposure in apple fruit. Several new putative α -amylase genes from apple and *Arabidopsis thaliana* were identified by Stanley *et al.* (2002), who suggested the presence of three families, each targeted to different compartments within the cell, perhaps having different substrate specificities.

Sorbitol has a unique role as an end product of photosynthesis in temperate fruit crops such as apple. Tao *et al.* (1995) examined sorbitol synthesis in transgenic tobacco with apple cDNA encoding nicotinamide adenine dinucleotide phosphate (NADP)-dependent sorbitol-6-phosphate dehydrogenase. Yamada *et al.* (1998) cloned NADP-dependent sorbitol from apple fruit and also examined gene expression. Hyperaccumulation of apple sorbitol-6-phosphate dehydrogenase expression in transgenic tobacco resulted in necrotic lesions (Sheveleva *et al.*, 1998). Sorbitol-6-phosphate phosphatase from apple leaves has also been purified and characterized by Zhou *et al.* (2003), who discussed a possible feedback mechanism for regulation of sorbitol biosynthesis.

Most plant MADS-box genes play a role in the development of floral meristems and organ identity. The MADS-box genes of apple were first investigated by Sung and An (1997), who cloned and characterized a MADS-box cDNA clone of 'Fuji' apple. Sung *et al.* (1999) characterized MdMADS2, a

member of the SQUAMOSA subfamily of genes in apple. Yao *et al.* (1999b) found that seven MADS-box genes in apple were expressed in different parts of the fruit. Jeong *et al.* (1999) cloned and characterized CONSTANS-like cDNA clones from 'Fuji' apple.

Kotoda *et al.* (2000) examined expression pattern of homologues of floral meristem identity genes *LFY* and *AP1* during flower development in apple, and then determined that MdMADS5 is a putative homologue of *Arabidopsis* APETA1 (*AP1*) and *Antirrhinum* SQUAMOSA. Overexpression of MdMADS5 caused early flowering in transgenic *Arabidopsis*. Five of 15 transformed plants flowered earlier and the flowers resembled *Arabidopsis*-expressing 35S:*AP1* or *tfl1* mutant plants, suggesting MsMADS5 may be involved in flower bud formation (Kotoda *et al.*, 2002). Wada *et al.* (2002) identified two orthologues of FLORICAULA/LEAFY involved in flowering.

Sung *et al.* (2000) examined developmentally regulated expression of two MADS-box genes – MdMADS3 and MdMADS4 – in the morphogenesis of flower buds and fruit in apple. Van der Linden *et al.* (2002) cloned and characterized four apple MADS-box genes isolated from vegetative tissue. Reverse transcription (RT)-PCR results showed that the three floral MADS-box genes (MdMADS13, 14 and 15) were expressed in vegetative tissues of adult and juvenile trees. MdMADS12, an *AP1*-like gene, was expressed at similar levels in leaves, shoots and floral tissues and its involvement in the transition from juvenile to adult was suggested.

Other genes involved in fruit development have been identified. Two putative plasma membrane intrinsic protein (PIP1) genes were isolated from apple. Research suggests that MdPIP1 is important in fruit expansion and in plants under osmotic stress (Hu *et al.*, 2003). Three cDNAs encoding spermidine synthase (SPDS), a key enzyme in polyamine biosynthesis, were cloned by Zhang *et al.* (2003). They determined that two cDNAs are spatially and developmentally regulated through alternative splicing and suggested that SPDS expression is regulated during apple fruit development.

2.2. Marker-assisted selection

Isozymes were the first markers to be investigated in apple. Their use resulted in the identification of linkages and they provided fingerprinting of scion and rootstock cultivars (Chyi and Weeden, 1984; Weeden and Lamb, 1985, 1987; Manganaris and Alston, 1987, 1988, 1992). Research continues with isozymes, as evidenced by their recent use in germplasm assessment (Itoiz and Royo, 2003) and genetic studies (Batlle and Alston, 1996; Chevreau *et al.*, 1999). Alston *et al.* (2000) reviewed isozymes and qualitative traits in apple and Tartarini and Sansavini (2003) reviewed the use of molecular markers in pome fruits. Luby and Shaw (2001) provided an excellent analysis of the advantages, disadvantages and economics of marker-assisted selection in fruit breeding.

Restriction fragment length polymorphisms (RFLPs) are co-dominant and more informative than randomly amplified polymorphic DNAs (RAPDs), but require more DNA than PCR-based methods. RFLPs are also labour intensive and difficult to automate. RFLPs have been used to a limited extent in apple to differentiate cultivars, to mark genes of interest and to fill in linkage maps. RFLP and RAPD markers linked to the *Sd1* gene conferring resistance to rosy leaf curling aphids (*D. devector* Wik.) were identified by Roche *et al.* (1997a), and a specific PCR assay was developed based on a RFLP marker closely linked to *Sd1* (Roche *et al.*, 1997b).

RAPD markers have been used most frequently in apple, but as new techniques become available they are being implemented. RAPDs have been used for genotyping and markers have been identified for fruit skin colour (Cheng *et al.*, 1996), for powdery mildew resistance (Markussen *et al.*, 1995; Dunemann *et al.*, 1999) and to verify apomictic seedlings from certain *Malus* species (Ur-Rahman *et al.*, 1997). The search for RAPDs linked to the *V_f* gene for resistance to scab was the objective of many researchers, including Gardiner *et al.* (1996), Yang *et al.* (1997) and Hemmat *et al.* (1998). RAPD markers have also been identified for scab resistance genes from Russian seedling

R12740–7A (Hemmat *et al.*, 2002) and from 'Antonovka' and Hansen's baccata no. 2 (Hemmat *et al.*, 2003b). Tremendous progress has also been made in the area of self-incompatibility in apple, from the initial work by Sassa *et al.* (1994, 1996) and Broothaerts *et al.* (1995) in sequencing the genes, to the development of a method for apple S-allele identification based on allele-specific PCR (Janssens *et al.*, 1995; Verdoodt *et al.*, 1998; Van Nerum *et al.*, 2001). These methods have been used to genotype cultivars (Kitahara *et al.*, 1997; Sakurai *et al.*, 1997; Komori *et al.*, 1998, 2000; Matsumoto *et al.*, 1999a,b; Matsumoto and Kitahara, 2000; Sakurai *et al.*, 2000; Van Nerum *et al.*, 2001; Kitahara and Matsumoto, 2002a,b) and to search for new incompatibility alleles. Research in this area has been reviewed by Broothaerts (2003) and Broothaerts *et al.* (2003). RAPDs have also been essential for the development of linkage maps (detailed below).

AFLPs may yield up to 30 polymorphic dominant markers per primer combination (Xu and Korban, 2000) and are very useful for extending existing genetic maps. Tignon *et al.* (2000, 2001a) used AFLPs for the identification of apple cultivars, but found that they could not be used reliably to differentiate among sports of a given cultivar. A set of two AFLP primers allowed the identification of 28 cultivars, with a total of 52 polymorphic bands. Zhu *et al.* (2001) demonstrated that *Pst*I-ACC/*Mse*I-GAC primers were useful for genotyping rootstocks. Goulao *et al.* (2001) compared AFLPs to RAPDs in discrimination and estimation of genetic similarities among apple cultivars and had consistent results with both methods; each identified the same four cultivars that were clustered and divergent from the other cultivars tested. AFLPs were used for fine-scale mapping for the *V_f* scab resistance gene (Xu and Korban, 2000, 2002) and for resistance to aphids (Cevik and King, 2000a).

Cevik and King (2002a) conducted a high-resolution genetic analysis of the *Sd*-1 aphid resistance locus in *Malus* spp. The *Sd*-1 gene confers resistance to *D. devecta* biotypes 1 and 2. Fine mapping was done using AFLPs. Two co-segregating AFLPs contained a common (GA)₂₃ repeat, from which a PCR-based

SSR assay was developed. The results suggested that *Sd*-1 and *Sd*-2 loci are tightly linked and probably allelic. Cevik and King (2002b) then resolved the *Sd*-1 locus on a BAC contig within a subtelomeric region of linkage group 7.

Xu *et al.* (2000) used a modified AFLP technique and the *Hpa*II and *Msp*I isoschizomers to detect DNA methylation in apple. When tested on DNA from samples from the field or from *in vitro* shoot cultures, approx. 25% of the AFLP bands were from methylated sequences, with a few bands unique to adult trees versus *in vitro* shoots.

Sequence-characterized amplified regions (SCARs) are increasingly being used. Cheng *et al.* (1998) developed a SCAR for the *V_m* gene for scab resistance. SCARs were developed from AFLPs for the *V_f* gene for scab resistance (Xu *et al.*, 2001a). The polymorphic DNA fragment OPAT20₄₅₀ linked to the PI1 powdery mildew (*P. leucotricha* (Ell. & Ev.) E.S. Salmon) resistance gene from *Malus* × *robusta* was cloned and sequenced and longer primers constructed for the generation of a SCAR marker (Markussen *et al.*, 1995). From seven AFLP markers, two SCARs (EM M01 and EM M02) were mapped at 4.6 and 6.4 recombination units from the powdery mildew resistance gene *Pl-w* from 'White Angel' (Evans and James, 2003). The SCAR marker SCB82670 developed by Kim, M.Y. *et al.* (2003) and linked to the *Co* gene is being studied for its utility in other populations segregating for *Co*.

SSR markers are co-dominant, abundant, highly polymorphic and reproducible. SSRs can be used to distinguish homozygotic and heterozygotic individuals. They are also very useful for aligning different linkage maps. However, the development of SSRs is labour intensive and can be expensive. Guilford *et al.* (1997) first examined the use of SSRs for cultivar identification. SSRs have also been very useful for studying diversity in *Malus*. Sixteen SSRs were used with 19 accessions and ten of these were mapped on an RAPD linkage map of 'Iduna' × A679/2 (Gianfranceschi *et al.*, 1998). Hokanson *et al.* (1998) used eight SSRs to analyse 66 accessions of *Malus* × *domestica*. The eight primers

chosen differentiated all but seven accessions. Hokanson *et al.* (2001) examined 142 *Malus* accessions using SSRs to determine genetic identities and estimated genetic diversity. SSRs developed from *Malus* × *domestica* were used successfully on different *Malus* species.

Hemmat *et al.* (1997) end-sequenced an RAPD marker identified as having the closest linkage to the *Co* gene, which imparts columnar or the reduced branching habit. An SSR of (GA)₁₇ was identified within the DNA fragment. Four allelic forms, including a null allele, were identified, with the null allele having the closest linkage with *Co*.

Liebhart *et al.* (2002) reported on the identification and use of 140 new SSRs, with 115 mapped on the 'Fiesta' × 'Discovery' linkage maps (detailed below). Hemmat *et al.* (2003b) used 41 sets of SSR primers to identify homologous linkage groups in maps of 'Golden Delicious', 'Liberty' and 'Wijcik McIntosh'.

SSRs can also be used to analyse putative homozygous lines by selecting only SSR primers that amplify different contrasting alleles. Hofer *et al.* (2002) obtained haploids in apple using anther culture and *in situ* parthenogenesis followed by *in vitro* culture of embryos or cotyledons. SSR analyses confirmed that the lines examined were homozygous.

In the future, SSRs and other markers may be useful for examining the synteny between maps in *Prunus*, *Pyrus* and *Malus*. Some SSRs isolated from apple identified polymorphism and genetic diversity in pear (Yamamoto *et al.*, 2001), but in some cases the allele size was different (Hemmat *et al.*, 2003b).

Inter-simple sequence repeats (ISSRs) describe a new method in which SSRs are used as primers to amplify mainly the inter-SSR regions (Reddy *et al.*, 2002). The primers are based on SSR sequences anchored to genomic sequences flanking each side of the targeted SSRs (5' or 3') by using two to four arbitrary or degenerate nucleotides. This technique does not require prior sequence information and it generates a high number of polymorphisms. ISSRs are thought to be of particular use in studying closely related

individuals that have low levels of polymorphism. Goulao and Oliveira (2001) characterized apple cultivars using SSR and ISSR markers and found them to be more reproducible than RAPDs and AFLPs.

ESTs have been generated from randomly selected clones of cDNA libraries prepared from diverse apple material. Young fruits, peel of mature fruits and carpels of 'Fuji' were targeted by Sung *et al.* (1998). Gardiner *et al.* (2003) discussed EST research and a candidate gene approach. More laboratories are researching ESTs in apple, but have not yet published their results.

Transposable elements are also being examined in apple. Tignon *et al.* (2001b) cloned fragments from a differential display of 'Jonagold' and its coloured sports. One of the fragments had strong sequence homology with the reverse transcriptase gene of several copia-like retrotransposons. Numerous copies were found in all the cultivars tested.

Two short repeat sequences were identified within the 5' flanking region of the ACC synthase genes. These apple repetitive sequences, designated Ars1 and Ars2, were present in the promoter of the Md-ACS1-1 ACC synthase gene. Amplification with primers designated from Ars1 and Ars2 revealed that both elements are present in high number in the apple genome and similar elements are present across the *Rosaceae*. Hadonou *et al.* (2003) suggested that these elements could be useful for genotyping.

Wakasa *et al.* (2003) discovered a mobile element when sequencing DNA marker fragments linked to fruit skin colour. One hundred and sixty-three base pairs in one fragment were classified as a mobile element due to target site duplication. This element was found in the 5' flanking region of six apple genes and has a copy number of 5000 to 6000 copies in the *Maloideae*. This miniature transposable element was named Majin.

Lee, S.Y. *et al.* (2003) compared superfamilies of nucleotide-binding sequence (NBS)-encoding disease resistance gene analogues (RGAs) in cultivated and wild apple species. They found that the RGA families were in both wild and cultivated species, but their sequences were divergent. The low level of

recombination was attributed to a gradual accumulation of mutations in the evolution of NBS-encoding sequences in apple.

The development of linkage maps has accelerated in apple. Hemmat *et al.* (1994) constructed the first linkage maps in apple using progeny of two apple cultivars, 'White Angel' × 'Rome Beauty'. Isozymes and RAPDs were used. The linkage map for 'White Angel' had 253 markers in 24 linkage groups and covered 950 cM. The 'Rome Beauty' map had 256 markers on 21 linkage groups. Thirteen linkage groups in 'Rome Beauty' were found to be homologous to 'White Angel'. Linkage maps for 'Wijcik McIntosh' and two scab-resistant (V_p) advanced breeding selections were developed by Conner *et al.* (1997), while Seglias and Gessler (1997) developed a map from progeny of 'Iduna' × A679-2. Gianfranceschi *et al.* (1998) mapped ten of 16 SSRs tested on the 'Iduna' × A679/2 linkage map and discussed the advantages of SSRs for integration of maps.

Development of the 'Prima' × 'Fiesta' linkage map resulted from a strong collaborative effort of researchers in Europe and New Zealand (Maliepaard *et al.*, 1998). The map was constructed using RFLPs, RAPDs, isozymes, SSRs and SCARs. Duplicate loci and segregation distortion were noted. The genes for scab resistance (V_p), resistance to rosy apple aphid (*Sd1*), self-incompatibility (*S*) and fruit acidity (*Ma*) were mapped.

Liebhart and Gessler (2000) stressed that, to construct a map with accurate marker location and consistent marker sequence for aligning linkage groups, markers must be of good quality, the population must be large enough and the mapping software must be understood. Common problems, such as out-cross individuals, thresholds in the mapping software and the quality of the markers were discussed relative to errors in mapping.

Liebhart *et al.* (2002) tested 140 SSRs with seven cultivars and one breeding selection. One hundred and fifteen were positioned on 'Fiesta' × 'Discovery', with at least two SSRs per 17 chromosomes, allowing the alignment of this map with other linkage maps in apple. Next, a saturated reference map for apple was constructed using the progeny

from a 'Fiesta' × 'Discovery' cross. A total of 840 molecular markers (475 AFLPs, 235 RAPDs, 129 SSRs and one SCAR) were used to construct the map. The large number of co-dominant markers allows a transfer of this map to other populations (Liebhart *et al.*, 2003b).

QTL studies are increasing in interest and importance. Lawson *et al.* (1995) used molecular markers to analyse the inheritance of morphological and developmental traits in apple. Branching habit, vegetative bud break, reproductive bud break, bloom time and root suckering were studied. The first detailed QTL study in apple using molecular markers was that of Conner *et al.* (1998), who used RAPDs to estimate the position and effects of QTLs for traits influencing juvenile tree growth and development in a population derived from 'Wijcik McIntosh' by a scab-resistant selection. Conner *et al.* (1998) also examined the limitations of dominant markers and an outbred mapping population for QTL analyses. Liebhart *et al.* (2003a) examined quantitative physiological traits in the 'Fiesta' × 'Discovery' progeny and identified a number of QTLs affecting stem diameter, plant height increment, leaf size, bloom traits, juvenile phase and fruit characteristics.

In a study of fruit firmness, QTLs for fruit quality traits were targeted (King *et al.*, 2000). Measurements of firmness included Magness-Taylor penetrometer readings, stiffness by acoustic resonance and sensory testing with a trained panel. Significant QTLs were detected for seven linkage groups with large effects on linkage groups 1, 10 and 16 of 'Prima' × 'Fiesta'. These studies were extended to examine variation in crispness and juiciness using wedge fracture and compression tests (King *et al.*, 2001). Significant QTLs for wedge fracture tests were detected on linkage groups 16 and 1, corresponding with positions identified by King *et al.* (2000) for sensory measures of crispness and juiciness.

The maximum likelihood interval mapping method was used to identify eight genomic regions associated with quantitative field resistance to apple scab. Six conferred resistance to foliar scab, and two conferred

resistance to the fruit (Liebhard *et al.*, 2003c). Given the interest in disease resistance, more QTL studies will follow, as evidenced by reports by Calenge *et al.* (2004).

2.3. Functional genomics

The study of scab resistance progressed from mapping genes to fine mapping to the discovery that apple contains receptor-like genes homologous to the *Cladosporium fulvum* resistance gene family (Vinatzer *et al.*, 2001), which are clustered in two linkage groups (Bus *et al.*, 2004; Hemmat *et al.*, 2003a). BAC libraries have been developed for 'Florina' (Vinatzer *et al.*, 1998), for *M. floribunda* 821, the source of the V_f scab resistance gene (Xu *et al.*, 2001b) and for 'Goldrush'. The V_f gene has been located and cloned (Patocchi *et al.*, 1999a,b; Xu and Korban, 2002). The transformation of susceptible cultivars with the candidate *HcrVf2* gene and determination that the transformants are resistant to scab is further progress towards confirming V_f gene identity and function (Barbieri *et al.*, 2003). Further research is needed to determine the effectiveness of this gene and that of the other homologues of *C. fulvum* resistance genes as well as the effect of copy number.

3. Micropropagation

Micropropagation of apple has been reviewed by Korban and Chen (1992), De Bondt *et al.* (1996) and Hammerschlag (2000). This section will discuss some of the most recent research.

Micropropagation of rootstocks must be economical and must not result in juvenile characteristics or increase the propensity of a clone to burr-knot production. A xyloglucan isolated from the seeds of *Hymenaea coubari* has been used for micropropagating 'Jonagold' and 'Marubakaido' rootstock (Lima-Nishimura *et al.*, 2003). Plants on gels created with a blend of 0.4% agar and 0.2% xyloglucan (w/v) had higher growth and multiplication and lower occurrence of hyperhydric shoots than plants grown on the

standard semi-solid medium. Even in the absence of auxin, shoot rooting was nearly 70% for 'Marubakaido' and 66% for 'Jonagored' on the modified medium, versus 6.7% and 10.4% on the standard semi-solid medium.

Chakrabarty *et al.* (2003) micropropagated 'M.9 East Malling Long Ashton (EMLA)' rootstock in a bioreactor. They outlined methods to reduce the occurrence of hyperhydricity of apple shoots that can occur when shoots are cultured in liquid medium. This research is a prerequisite for use of bioreactors as a cost-effective commercial micropropagation method.

Rooting plants *in vitro* has been a challenge. Ma *et al.* (1998) found that ethylene inhibitors promoted root formation in apple shoot cultures. The positive effects may result from a reduction of the ethylene concentration or inhibition of ethylene action. Calamar and de Klerk (2002) examined the effect of sucrose on adventitious root regeneration in microcuttings and stem slices from microcuttings. Sucrose influenced the number of adventitious roots, but at a broad range of concentrations (1–9%) the effect was small. Increasing the sucrose concentration shifted the dose–response curve of auxin to the right. Sucrose was most important during the initial rooting period. Ricci *et al.* (2003) determined that weakly cytokinin-active diphenylurea derivatives influenced adventitious rooting in 'M.26'. When triacontanol was added to a multiplication and rooting medium, it increased the number and the fresh weight of shoots of JTE-E4 in the multiplication phase and enhanced root number and chlorophyll content in the rooting phase (Tantos *et al.*, 2001).

Diaz-Perez *et al.* (1995) examined acclimatization and subsequent gas exchange, water relations, survival and growth of micropropagated plantlets after transplanting to soil. High plant water status, as indicated by high relative water content, was an important factor for plant survival and growth. Bolar *et al.* (1998) developed a method for rooting and acclimatization of micropropagated apple shoots. Shoots were placed in root induction medium with indolebutyric acid (IBA) for 1 week in the dark, followed by transfer to the

light and medium without IBA for root elongation. Rooted shoots were transferred to Jiffy-7s supplemented with a biological plant protectant and fertilizer and incubated in plastic humidity trays. After 2–3 weeks plants were transferred to pots and covered with plastic bags. This technique resulted in 70–100% survival of shoots. While mycorrhizal inoculation and acclimatization of micropropagated rootstocks had a positive effect on root and shoot growth of ‘Marubakaido’ rootstock, it had a negative effect on the growth of ‘M.9’ (Locatelli and Lovato, 2002). Lane *et al.* (2003) developed a protocol to micrograft shoot tips from *in vitro* cultures directly to transplanted rootstock plants, thus eliminating the rooting stage. They suggested that this procedure could be used to propagate transgenic clones for field-testing.

Zhu *et al.* (1999) looked at growth rates and biomass production of micropropagated ‘M.26’ and ‘Gravenstein’ on their own roots and in different micrografted combinations under non-limiting and limiting nutrient conditions. Their results suggested that the dwarfing effect was not directly related to the relative growth rate of the rootstock or scion, but instead was associated with root morphology and the ability of roots to absorb nutrients.

The orchard performance of trees from micropropagules was examined by Zimmerman and Steffens (1995), who measured the effect of cultivar, planting density and plant growth regulators on growth and fruiting of own-rooted micropropagated ‘Gala’ and ‘Triple Red Delicious’. Growth regulators were unable to dwarf trees sufficiently. The development of burr-knots on all ‘Gala’ trees in this trial was a concern and the authors suggested that *in vitro* cultures ought to be established yearly for cultivars prone to burr-knot formation.

Zimmerman (1997) evaluated orchard variation in micropropagated trees of ‘Redspur Delicious’ apple. Micropropagated trees lacked uniformity in tree size, appearance and flowering. When shoots from spur-type trees were recultured the resulting trees were uniform in appearance. Zimmerman suggested that when micropropagating spur-

type apples the source should be previously micropropagated trees that maintained clonal fidelity. This might overcome a potential problem with clonal variation in orchard planting of spur-type micropropagated trees.

4. Micrografting to Eliminate Viruses

Viruses are a problem in apple, and are spread by grafting. Meristem culture, with or without heat treatment, has been used to obtain virus-free clones. The role of tissue culture in international exchange and quarantine of germplasm in the USA and Canada has been reviewed by Salih *et al.* (2001). Advances in detection of viruses have occurred, especially in the area of RT-PCR assays. James *et al.* (1997) eliminated apple stem-grooving virus by chemotherapy and developed an immunocapture RT-PCR for rapid and sensitive screening. A rapid and homogeneous detection system for apple stem pitting virus using RT-PCR and a fluorogenic 3′ minor groove binder DNA probe has been reported (Salmon *et al.*, 2002). This assay correlates 96% with gel analysis and is more reliable than biological indexing. Multiplex RT-PCR–enzyme-linked immunosorbent assay (ELISA) is more reliable than the use of indicator plant bioassays for detecting apple chlorotic leaf spot, apple stem-pitting, apple mosaic and apple stem-grooving viruses (Menzel *et al.*, 2003).

5. Somatic Cell Genetics

5.1. Regeneration

5.1.1. Somatic embryogenesis

Induction. Induction of embryogenic cultures from the nucellus of immature apple seeds was reported by Eichholtz *et al.* (1980) and James *et al.* (1984). The protocol of James *et al.* (1984) involved the use of tissues from immature apple seeds at 30 and 50 days after anthesis. The nucellus, the endosperm and the zygotic embryo were extracted and cultured separately. Nucellar tissues from 30-day-old seeds formed embryogenic cultures

only in the presence of either 2,4-dichlorophenoxyacetic acid (2,4-D) or naphthaleneacetic acid (NAA) together with benzyladenine (BA). Concentrations of 2 μ M 2,4-D or 2.6 μ M NAA and 4.4 μ M or 2.2 μ M BA gave the best response. For most cultivars tested, Linsmaier and Skoog (1965) (LS) medium was the optimum medium, but for two rootstocks ('M.9' and 'M.27') Murashige and Tucker (1969) (MT) medium produced the highest frequencies of adventitious embryos.

Maintenance. Factors affecting secondary somatic embryogenesis in 'Gloster 69' included explant source, e.g. cotyledon-derived cultures of immature zygotic embryos, somatic embryo size, type and concentration of carbohydrates and gelling agents (Daigny *et al.*, 1996). Secondary somatic embryogenesis was optimized on medium containing either a combination of 5.3 μ M NAA, 0.9 μ M BA and 0.9 μ M kinetin or 10 μ M thidiazuron (TDZ) alone, 175 mM maltose and 2.8 g/l Phytagel.

Development. Primary explants were cultured on basal medium supplemented with various combinations of plant growth regulators. Cultures were maintained at $25 \pm 1^\circ\text{C}$ in darkness and subcultured on to fresh regeneration medium at 3-week intervals for an additional 2 months (Daigny *et al.*, 1996).

Germination/conversion. The conversion treatment consisted of 2 months at $4 \pm 1^\circ\text{C}$ in darkness on half-strength Murashige and Skoog (1962) (MS) medium for dormancy removal and subculture on fresh medium under a 16 h light photoperiod (20 $\mu\text{mol}/\text{m}^2/\text{s}$) at 25°C for root and shoot development (Daigny *et al.*, 1996).

5.1.2. Organogenesis

Rugini and Muganu (1998) induced shoot formation from callus derived from established shoots of 'Golden Delicious' on a medium formulation of macroelements consisting of 950 mg/l KNO_3 , 230 mg/l NH_4NO_3 , 580 mg/l $\text{Ca}(\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}$, 370 mg/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 270 mg/l KH_2PO_4

MS microelements and iron and 0.45 μ M TDZ with 0.54 μ M NAA. This technique involved adventitious bud production from cultured primary leaves from established shoot cultures. Leaflets of 2–3 mm in length were critical for production of morphogenic callus. Bommineni *et al.* (2001) also reported induction of shoot organogenesis of 'Gala Gala' from preconditioned, micropropagated shoots, by sectioning pre-treated stems into thin slices and culturing them under either light or dark conditions. Approximately 37% of the cultured stem slices resulted in shoot induction. Medium supplemented with 9.08 μ M TDZ was superior to medium supplemented with BA and kinetin.

Liu *et al.* (1998) etiolated 'Royal Gala' to promote high-frequency shoot organogenesis. When the first or youngest internodal explants from etiolated shoots were cultured, the yield of shoots was two-, eight- and 73-fold higher than the second, third and fourth internodal explants, respectively. These explants also produced a sevenfold increase in shoots compared to similar explants from non-etiolated shoots. Yancheva *et al.* (2003) found that the auxin type and timing of its application and the length of exposure to the specific auxin were critical for induction.

5.1.3. Haploid recovery from anthers or ovules

Lespinasse and Godicheau (1980) reported haploidy in apple, and haploidy in apple and pear has been reviewed by Lespinasse *et al.* (1999) and Hofer and Lespinasse (1996). The three methods commonly used to obtain haploids are: (i) screening maternal haploid plants from seedlings (approx. 1% frequency); (ii) *in vitro* androgenesis using anther culture (<1% efficiency, although Zhang (1988) reported 6.6% with 'Topred'); and (iii) induction of *in situ* parthenogenesis by irradiated pollen. The last method results in approx. five embryos per 1000 flowers pollinated. Recovery of plants from androgenic embryos has been difficult (Lespinasse *et al.*, 1999). Spontaneous chromosome doubling has occurred with some haploids following several years of micropropagation.

De Witte and Keulemans (1994) evaluated restrictions of the efficiency of haploid production from 'Idared' by parthenogenesis *in situ*. Efficiency was affected by seed set, embryo germination and green plant recovery from germinated embryos. Green plant production was influenced strongly by irradiation dose, picking time and quality of irradiated pollen; induction efficiency was highest when seed weight was high. The effect of pollination techniques and plant growth regulators on fruit set and parthenogenesis was examined by De Witte and Keulemans (2000). *Ex vitro* germination of homozygous diploid plants was also studied. Use of a bee-booster system appeared to increase yield. Daminozide had a slightly positive effect on yield, but NAAm, an anti-auxin derivative, did not. For parthenogenic seed > 50 mg germination in the greenhouse and *in vitro* was similar and the number of putative homozygous plants was higher.

Keulemans and De Witte (2000) tried to improve the pollination stimulus for parthenogenic egg cell development. Although kaempferol, which stimulates pollen germination and growth, gave inconsistent results, repeated pollination positively affected embryo yield, but was affected by cultivar and year. Pollination with high-dose pollen followed by repollination with low-dose pollen also enhanced yield. King flowers gave a better embryo yield than laterals, and this was consistent across cultivars and years. Higher pollination temperatures also had a net positive effect. Verdoodt *et al.* (1998) successfully used PCR-based assays for the self-incompatibility alleles of parental cultivars to assess homozygosity in shoots obtained from haploids using irradiated pollen of 'Baskatong'.

Doubled haploids have been obtained by embedding haploid shoots in an agar solution containing oryzalin (Bouvier *et al.*, 1994). 'Remo' was used to obtain the first homozygous scab- and mildew-resistant doubled haploid lines (Hofer *et al.*, 1999a). Preliminary evaluation of doubled haploids obtained from anther and microspore culture and *in situ* parthenogenesis revealed that a single donor genotype could result in regen-

erants that were diploid, triploid and tetraploid, but 93% of the lines were homozygous as indicated by isozymes. Of 30 lines from scab-resistant donors, 24 had a marker for the V_f resistance (Hofer and Grafe, 2000).

5.1.4. Protoplast isolation and culture

Plants have been obtained from leaf mesophyll protoplasts of *in vitro*-cultured shoots of a columnar apple (Wallin and Johansson, 1989). Diekmann *et al.* (1999) studied the morphogenic response of six genotypes of apple from protoplasts. Donor material for protoplasts included young etiolated shoots and young mesophyll tissue from shoot cultures grown in darkness. Cell divisions were observed in all apple genotypes tested. Callus developed from protoplasts of five of the six genotypes and shoots regenerated from 'M.26', 'Gala' and 'Pinova' cultures. The preculture of the donor plants, especially the spectral composition of the light source, had a strong influence on success, as did the addition of sorbitol to the protoplast culture media.

Saito and Suzuki (1999) outlined a method for regeneration of 'Fuji' from meristem-derived callus protoplasts. Maddumage *et al.* (2002) reported the efficient transient transformation of suspension culture-derived apple protoplasts. Apple leaf protoplasts have been transformed with a construct containing a viral leader and plant enhancer linked to a promoter to enhance expression of the human respiratory syncytial virus F gene (Sandhu *et al.*, 1999).

5.2. Genetic manipulation

5.2.1. Mutation induction and somaclonal variation

Mutation breeding in vegetatively propagated crops has been reviewed by Van Harten (1998) and Predieri (2001). Predieri (2001) contrasted the number of publications on mutation breeding with the number of released cultivars developed by mutation, and concluded that mutagenesis coupled

with tissue culture is either ineffective or not exploited fully in fruit crops. Lacey and Campbell (1987) reported on the selection, stability and propagation of mutant lines of apple. When mutations occur, either spontaneously or induced, the change must encompass the entire histogenic layer for propagules to be uniform. Fruit skin colour changes have mutations in the epidermis or LI layer. Only changes in LII are useful in breeding, since gametes are formed in this layer. The type of mutations that occur in apple and the occurrence of chimeras have been reviewed by Pratt (1983).

Adequate fruit skin coloration is important for marketing red-skinned apples. There are many naturally occurring mutations for greater fruit skin colour. Such mutations are commercialized if they are stable (periclinal) and if no other varietal characteristics have been altered. 'Delicious', 'McIntosh', 'Fuji', 'Braeburn' and 'Gala' are prone to mutations for greater skin colour, and many genetic sports are offered by nurseries. These mutations provide greater numbers of fruit that are highly coloured and of a higher grade, but reversion to poorly coloured fruit can occur, especially with cultivars known to be prone to colour mutations.

Red colour changes in fruit skin colour of 'Royal Gala' have been studied following irradiation of apple scions (White *et al.*, 1994). McMeans *et al.* (1998) assessed *in vitro*-cultured 'Gala' and 'Royal Gala' from axillary and adventitious buds and observed little somaclonal variation with respect to fruit colour.

Donovan *et al.* (1994a,b) assessed somaclonal variants of 'Greensleeves' for resistance to the fire blight pathogen *E. amylovora* and for rooting ability and shoot proliferation *in vitro*. Thirty-three per cent of the 270 somaclones showed increased resistance to *E. amylovora* and there was considerable variation in rooting ability, root number and rate of shoot proliferation. Chevreau *et al.* (1998) examined fire blight resistance and genetic trueness to type of four somaclonal variants from the apple cultivar 'Greensleeves'.

Selecting apple rootstock somaclones for tolerance of aluminium has been investi-

gated (Dantas *et al.*, 2001). Resistant rootstocks would be useful for soils with pH below 5 where toxic levels of aluminium affect calcium absorption, with a negative effect on fruit quality. Three groups were identified based on a reduction of the percentage of dry matter of the shoots. Clones identified had either no tolerance, slight tolerance or tolerance of aluminium.

The use of apple fruit cells to investigate physiological processes is a way to study responses to culture and stresses. Bowen *et al.* (2002) found that the heat-shock response is involved in thermotolerance in suspension-cultured apple fruit cells.

5.2.2. Somatic hybridization

Symmetric and asymmetric protoplast fusion has been used to produce somatic hybrids in apple. Symmetric protoplast fusion produced some somatic hybrids and asymmetric hybridization produced some putative hybrids that need to be analysed further (Huancaruna Perales *et al.*, 2000).

5.2.3. Genetic transformation

Breeding objectives. The use of genetic transformation in apple has a tremendous advantage of maintaining cultivar identity. A key characteristic, such as disease resistance, can be changed and yet the phenotype stays the same. Name recognition is important in marketing apples. The self-incompatibility and inbreeding depression that exists in *Malus* prevents us from using a back-cross technique to introgress genes. We can introduce genes for resistance, but, because we must cross back to a different cultivar each time, cultivar identity is lost and a new hybrid is produced. Objectives in genetic transformation include: imparting resistance to diseases and pests, modifying fruit softening, understanding genes involved in flowering and fruiting and using antisense technology to silence genes. Objectives for the transformation of rootstocks are similar, but also include genes for dwarfing, resistance to viruses and genes influencing rooting and propagation.

Protocol. Singh and Sansavini (1998) reviewed transformation for several fruit crops, while Hammerschlag (2000) reviewed transformation of *Malus*. De Bondt *et al.* (1994, 1996) reviewed factors influencing gene transfer efficiency during early transformation steps with *Agrobacterium tumefaciens* and factors affecting regeneration of transformants. Maximova *et al.* (1998) investigated transformation using green fluorescent protein and found that high transient expression and low rates of stable transformation suggested that factors other than transferred-DNA transfer were rate limiting.

The growth stage of the *Agrobacterium* cells was found to be important in increasing the efficiency of transformation of 'Gala' and 'McIntosh Wijcik' (Song *et al.*, 2001). Regeneration and transfer efficiency were highest using *Agrobacterium* cells of optical density OD₆₀₀ 0.9–1.1 incubated at 28°C for 24 h in liquid YEB medium. While biolistic transformation has not been a major emphasis with apple, Gercheva *et al.* (1994) found that particle bombardment of apple leaf explants influenced adventitious shoot formation.

Accomplishments. Apple was first transformed by James *et al.* (1989). Trifonova *et al.* (1994) transformed 'Granny Smith' with neomycin phosphotransferase (*nptII*) and *ipt* genes, encoding for one of the first enzymes in the cytokinin biosynthetic pathway. Yao *et al.* (1995) introduced the acetolactate synthase gene into 'Royal Gala' to increase resistance to the herbicide 'Glean[®]' in transgenic plants. James *et al.* (1996) documented the stable expression and Mendelian segregation of the marker transgenes nopaline synthase (*nos*) and the co-transferred gene *nptIII* in the flesh of apple fruits 7 years after the initial transformation. 'Gala', 'Golden Delicious' and 'Elstar' were transformed (Puite and Schaart, 1996), followed by 'Delicious' and 'Pink Lady' (Sriskandarajah and Goodwin, 1998) and 'Delicious', 'Greensleeves' and 'Royal Gala' (Maximova *et al.*, 1998).

Bolar *et al.* (1999) developed an efficient transformation system for 'Marshall McIntosh'. Expression of endochitinase from *Trichoderma harzianum* in apple increased resistance to scab and reduced vigour in

transgenic 'Marshall McIntosh' (Bolar *et al.*, 2000). There was a significant negative correlation between the level of endochitinase production and both the amount of disease and plant growth. Bolar *et al.* (2001) found that there was a synergistic activity of endochitinase from *Trichoderma atroviride* (*T. harzianum*) against apple scab in transgenic plants.

Yao *et al.* (1999a) grew transgenic 'Royal Gala' apple trees under controlled greenhouse conditions and 20% of the trees flowered in the second year, but when scion wood from the top of these clones was grafted on 'M.9' rootstock, 85% produced flowers and fruit the next year. Inheritance of three transgenes, *uidA*, *nptIII* and acetolactate, fit a 1 : 1 ratio in most lines, but in one progeny line the T-DNA integration pattern was complex.

Two of four transgenic lines possessing the kanamycin resistance gene and antisense PPO DNA showed repressed PPO activity and a lower browning potential than control shoots (Murata *et al.*, 2000). Broothaerts *et al.* (2000b) developed a spectrophotometric assay for the analysis of PPO in apple and tobacco leaves to increase efficiency in screening large numbers of transgenic plants.

Transformation of 'Jonagold' with antimicrobial peptide genes (A1-AMP) resulted in 28 independent transgenic lines, which are being tested for resistance to apple scab using artificial inoculation assays (Broothaerts *et al.*, 2000a). At the Apple Research Centre in Morioka, Japan, 'Orin' and the Japanese rootstock 'JM 7' have been transformed with genes encoding the sorbitol-metabolizing enzyme, sorbitol-6-phosphate dehydrogenase isolated from apple, chitinase isolated from rice, glucanase from soybean and sacrotoxin from the flesh-fly (Soejima *et al.*, 2000).

Non-target effects must also be examined in transgenic lines. Over-expression of PG in transgenic apple trees has resulted in a range of novel phenotypes involving changes in cell adhesion (Atkinson *et al.*, 2002), including silver leaves and non-functioning guard cells. Bolar *et al.* (2000) found that expression of endochitinase from *T. harzianum* in trans-

genic apple increased resistance to apple scab and reduced vigour.

Chevreau *et al.* (2001) transformed both a susceptible ('Galaxy') and a scab-resistant (x6407) apple cultivar with a purindole gene to determine if a resistant cultivar would confer greater resistance when coupled with a transgene. Barbieri *et al.* (2003) transformed apple to help verify the function of the cloned gene believed to be the V_f gene from *Malus floribunda*.

Use of lytic peptides to impart resistance to bacterial diseases such as fire blight (*E. amylovora*) in scion cultivars such as 'Galaxy' was reviewed by Aldwinckle *et al.* (2003). Ko *et al.* (1999) developed a method to quantify attacin expression in transgenic lines and then found that T4 lysozyme and attacin genes enhanced resistance of transgenic 'Galaxy' apple against *E. amylovora* (Ko *et al.*, 2002). The effect of an untranslated leader sequence was also examined by Ko *et al.* (2000). Transformation with a modified cecropin gene was the focus of research by Liu *et al.* (2001).

Szankowski *et al.* (2003) transformed the apple cultivars 'Elstar' and 'Holsteiner Cox' with the stilbene synthase gene from grape (*Vitis vinifera* L.) and with a polygalacturonase-inhibiting protein (PGIP) gene from kiwi (*Actinidia deliciosa*) to impart fungal resistance. A total of 13 transgenic lines were obtained, with some lines showing evidence of gene silencing. Broothaerts *et al.* (2003) used S ribonuclease (S-RNase) gene silencing to obtain self-fertile transgenic lines.

Wilson and James (2003) reported transformation of 'Queen Cox', an important apple cultivar in the United Kingdom. Research on down-regulation of ACC synthase and ACC oxidase to reduce softening, down-regulation of gibberellin 20 (GA20) oxidase to impart dwarfing and vaccine production in apple were also discussed (James *et al.*, 2003). Markwick *et al.* (2003) examined the influence of biotin-binding proteins to impart resistance to light brown moth (*Epiphyas postvittana* (Walker)) in apple.

Hvarleva *et al.* (2002) transformed 'Granny Smith' and 'M.26' rootstock with developmentally related genes (Knap1 and small auxin up-regulated RNAs (SAUR)). All

Knap1 transformants had altered morphology and growth, suggesting Knap1's role in the development of leaves and stems in apple. SAUR transgenic lines did not appear to have any morphological alterations.

Transformation of rootstocks has also been reported, with many different clones being targeted. The dwarfing rootstock 'Malling 26' ('M.26') was first transformed by Lambert and Tepfer (1992) and by Maheswaren *et al.* (1992). Holefors *et al.* (2000) introduced the *Arabidopsis* phytochrome B gene into 'M.26' and found no effect on rooting, but stem length was reduced in nine out of 13 lines. Shoot, root and plant dry weight were reduced in all transformants.

Transformation of the rootstock 'M.7' was first achieved by Norelli *et al.* (1994). Alterations in *nptII* and glucuronidase (*gus*) expression following micropropagation of transgenic 'M.7' apple rootstock lines suggested that gene silencing had occurred in some lines, as evidenced by methylation, whereas other lines might be comprised of transformed and non-transformed cells (Ko *et al.*, 1998).

The *rol* genes from *Agrobacterium rhizogenes* have been popular transgenes for rootstocks. Holefors *et al.* (1998) transformed 'M.26' with the *rolA* gene and examined this transgene's influence on growth. 'M.26' was transformed with both *rolA* and *rolB* by Zhu and Welander (2000). Zhu *et al.* (2001a) found that the introduction of the *rolA* reduced height and shortened internodes in the vigorous 'A2' rootstock. Two transgenic lines are under test. Zhu *et al.* (2001b) transformed 'M.9' with *rolB* and found that all of the transgenic clones rooted (83–100%) on hormone-free rooting medium *in vitro* versus 1% for the controls. Root length and root morphology did not differ between the transgenic clones and the untransformed controls.

'Jork 9' rootstock transformed with the *rolB* had increased root percentage and root number, with 13.8 roots per shoot versus 2.3 for the untransformed control. However, copy number had a large effect, with more than two copies of *rolB* reducing the number of roots and the number of rooted shoots (Sedira *et al.*, 2001).

Igarashi *et al.* (2002) introduced *rolC* into 'Marubakaidou' to obtain new dwarf cultivars with high rooting ability from cuttings. Transgenic lines had one to three copies of the *rolC* transgene. Four phenotypic groups of *rolC* transformants were identified. One group had reduced height and shortened internodes and another group had reduced height and normal internodes. The third group had normal height and yet had shortened internodes while the fourth group was phenotypically equivalent to the control plants. There was no correlation between phenotype and transcriptional activity of the *rolC* gene.

Resistance to fire blight continues to be an objective in rootstocks. Aldwinckle *et al.* (2003) reviewed transformation to impart resistance to fire blight. Gene silencing of *Agrobacterium* oncogenes was used to produce crown gall-resistant transgenic apple trees (Viss *et al.*, 2003). Transgenes designed to express double-stranded RNA from *iaaM* and *ipt* sequences prevented crown gall disease on the roots of transgenic apple.

The ability to restrict transgene expression to the tissues requiring the encoded activity is very beneficial. Gittins *et al.* (2000) looked at the effect of heterologous ribulose-1,5-biphosphate carboxylase/oxygenase small subunit (SSU) gene promoters on transgene expression in apple vegetative tissue. The promoters RBCS3CP from tomato (*Lycopersicon esculentum* Mill) and SRS1P from soybean (*Glycine max*) were examined. SSU promoters were active primarily in green vegetative tissues. Mean GUS activity was about half that with the cauliflower mosaic virus (CaMV) 35S promoter. The activity of SRS1 was dependent on light, while RBCS3C did not appear to be light dependent. GUS activity was localized in mesophyll and palisade cells of the leaf. Gittins *et al.* (2000) concluded that both SSU promoters would be suitable for transgene expression in photosynthetic tissues.

The *Brassica napus* extA promoter was examined as a means to have root-specific expression of transgenes (Gittins *et al.*, 2001); however, only a portion of the transgenic lines obtained contained the entire promoter,

with many having deletions. In addition, GUS staining indicated that young stem tissues and load-bearing areas had transgene expression, indicating that this promoter is not root-specific in apple.

James *et al.* (2001) examined tissue-specific transgene expression using seven heterologous and five homologous promoters. The vascular specific promoters, *rolC* and Commelina yellow mottle virus promoter (CoYMV) were on average only 20% of the CaMV 35S values, when expressed as means across all tissues. Fruit-specific promoters and the ethylene-inducible promoters β -galactosidase and ACC synthase have been cloned from an apple genomic library using cDNA sequences as probes. Patent applications have been filed for these promoters.

Transgene expression driven by the vascular-specific *rolC* and CoYMV promoters was examined in apple vegetative tissues by Gittins *et al.* (2003). Both promoters expressed *gusA* at a lower level than the CaMV 35S promoter. With both the *rolC* and CoYMV promoters, reporter gene activity was primarily in the vascular tissues, especially the phloem.

Progress is being made in the effective use of transgenes; however, traits that are complex, e.g. yield and flavour, are not likely candidates for improvement by biotechnology. In addition, there is a need for genes from *Malus* to be cloned, as public concern about transgene technology does differentiate between native and non-native genes. There is a need for specific promoters, wound-inducible or fruit- or leaf-specific, so that gene expression may be targeted only to the parts of the plant necessary for the desired effect. Transgenic testing must ensure that there are no non-target effects and that transgenic lines are stable and non-chimeric.

While there are still many restrictions associated with field-testing of transgenic apple lines, progress is being made. Caprio and Suckling (2000) examined the issue of cross-resistance between *Bacillus thuringiensis* (Bt) toxins in transformed clover and apples in New Zealand. Additional studies are likely to follow.

5.3. Cryopreservation

Maintaining material *in vitro* under slow growth conditions could reduce costs associated with less frequent subculturing and reduce opportunities for mislabelling and contamination. Negri (2000) studied slow growth of single node shoots of apple genotypes. Up to 2500 propagules/m² can be stored in microvessels at 2°C. Hao and Deng (2003) examined AFLP banding patterns of 'Gala' shoot tips that were stored *in vitro* for 1 year using low-temperature slow growth. There were no differences between samples before and after storage; however, there was a significant DNA methylation change in stored shoots compared to the samples prior to storage.

Brischia *et al.* (2002) encapsulated differentiating propagules of 'M.26' rootstock as synthetic seed. Encapsulated organogenic explants prepared by hand versus machine-prepared explants were compared. Hand-cut uninodal cuttings had 25% conversion to plantlets as opposed to only 11% for machine-cut explants. Higher yields are essential for commercial use of this technology.

Sakai and Nishiyama (1977) first addressed cryopreservation of dormant vegetative buds of hardy fruit trees in liquid nitrogen. Forsline *et al.* (1998) studied recovery and longevity of cryopreserved apple buds, while Stushnoff (1991) reviewed cryopreservation and the implications for maintenance and diversity during conservation. Forsline *et al.* (1999) detailed base and active collections of cryopreserved dormant apple buds in a germplasm repository. The hardiness of germplasm was addressed by Seufferheld *et al.* (1999) in a study involving cryopreservation of cold-tender apple germplasm.

The importance of the cryopreservation technique and preconditioning of the donor material to increase the likelihood of success was stressed by Wu *et al.* (1999). Axillary shoot tips of 'Golden Delicious' were successfully cryopreserved using an encapsulation–dehydration or encapsulation–vitrification technique, the latter without a cold-hardening pre-treatment (Paul *et al.*, 2000). The encapsulation–dehydration protocol allowed easier

manipulation of organs by alginate embedding, resulted in a higher percentage of cryopreserved shoots with regrowth (64 to 77%, depending on the cultivar), and had a simple freeze–thaw procedure.

Research is still needed on the extent, if any, of somaclonal variation in cryopreserved buds, especially in cultivars prone to mutations for fruit skin colour, e.g. 'Gala' and 'Fuji', for russetting ('Golden Delicious') or for spur-type plant habit ('Delicious' and other cultivars).

6. Conclusions

Prospects for the genetic improvement of apple have never been better. We have diverse germplasm with traits that can be used in breeding or in transgenic approaches. There is a better understanding of the most important diseases and insects and the genes conferring resistance. Advances in breeding for resistance and for quality are anticipated using candidate gene mining and using sequences from other well-characterized plant genomes. Quality traits are better understood and will be enhanced through the interdisciplinary approach of many programmes. Plant form, growth and development will be accelerated as genes from apple are characterized and their expression revealed.

The synteny between *Pyrus* and *Malus* maps (Chevreau *et al.*, 1999; Yamamoto *et al.*, 2001) will aid mapping and cloning efforts, as will the availability of consensus and saturated linkage maps. Genes from apple have already been cloned and transformation has verified their expression. Additional genes from *Malus* will become available for use in transformation. New promoters will target expression to the areas of interest. Studies of transgene expression and inheritance will provide breeders with a chance to use transgenes in conventional breeding. Over-expression of genes and their effects will add to our understanding of complex traits. In the future, partnerships between industry and breeding programmes or biotechnology companies may be a means to provide long-term financial support for this research. One

example is the partnership between the group NOVADI and the Institut National de la Recherche Agronomique (INRA) apple breeding programme in France (Laurens and Pitiot, 2003). Apple breeding and biotechnology have benefited from the efforts of many individuals, groups and disciplines, and there is still much to be gained from collaboration on germplasm, primers, sequence information, promoters and transgenes. All material, whether from breeding or transformation, must be thoroughly evaluated before release. New high-quality apple cultivars will continue to be developed and many existing commercial apple cultivars will be improved by transgenic approaches.

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18.3 *Prunus* spp. Almond, Apricot, Cherry, Nectarine, Peach and Plum

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1. Introduction

1.1. Botany and history

Prunus, subfamily *Prunoideae*, is the most horticulturally valuable genus in the *Rosaceae* after *Malus*. *Prunus* species are described as stone fruits due to the encasement of the seed within a lignified stone-like endocarp. Common *Prunus* fruits include peach, nectarine (*P. persica* (L.) Batsch), European plum (*P. domestica* L.), Japanese plum (*P. salicina* Lindl.), sour cherry (*P. cerasus* L.), sweet cherry (*P. avium* L.), apricot (*P. armeniaca* L.) and the nut crop, almond (*P. dulcis* Miller). Interspecific hybridization between *Prunus* species occurs in nature and interspecific hybridization has been used to develop novel commercial fruit types. Most of the early *Prunus* cultivars were seedlings selected from open-pollinated natural populations. Domestication and cultivation of *P. persica* and *P. armeniaca* occurred in China between 3300 and 2500 BC (Faust and Timon, 1995).

There have been several recent reviews on the biotechnology of *Prunus* (Schuerman and Dandekar, 1993; Oliveira *et al.*, 1996; Sing and Sansavini, 1998; Rugini and Gutierrez-Pesce, 1999; Srinivasan and Scorza, 1999; Scorza, 2001).

sent Iran) and named the fruit *persica*, assuming Persia to have been the centre of origin of peaches. The Romans spread peaches throughout their empire (Hesse, 1975; Faust and Timon, 1995; Scorza and Sherman, 1996). Peaches were introduced to the New World following the Spanish conquest. All commercial peach cultivars belong to *P. persica*, a diploid ($2n = 2x = 16$). Other peach-like species include *P. davidiana* (Carr) Franch., *P. mira* Kochne, *P. ferganensis* (Kost. et Rjab) Kov. et Kost. and *P. kansuensis* Rehd. Fruits can be beaked, round, flat shaped, yellow, white, red, melting or non-melting fleshed; clingstone or freestone; and with smooth or pubescent epidermis. Flowers can be large, medium or small, white, pink, red or mixed. Tree growth habits include columnar, dwarf, spreading and weeping (Faust and Timon, 1995). Peach flowers are borne on 1-year-old shoots, with few spurs forming, although spur-type trees have been reported (Scorza, 1987). The peach fruit is a drupe with a thin epidermis and fleshy mesocarp, which may or may not be free from the stony endocarp. Hesse (1975) and Scorza and Sherman (1996) summarized the botany and breeding techniques for peach.

1.1.2. Plum

There are three major cultivated plum groups, which were independently domesticated on different continents. There are 20 to

1.1.1. Peach

China is the centre of origin of peaches. The Romans acquired the peach from Persia (pre-

40 species of plum, with diploid species being relatively cross-compatible (Okie and Weinberger, 1996). Many plums are self-incompatible. The hexaploid ($2n = 6x = 48$) European plum (*P. domestica*) is adapted to cooler regions, and is the most common cultivated plum worldwide. The Asian or Japanese plum (*P. saliciana*) is a diploid ($2n = 2x = 16$) that originated in China and resulted from inter-species crosses. The North American plum species, *P. angustifolia*, *P. maritime* and *P. subcordata*, are found in isolated areas of the USA and Canada. Plum flowers are small but showy. They have 20–30 stamens, which, along with five petals and sepals, are attached to the rim of the calyx cup. Plums are distinguished from peach and almond by an elongated pedicel, and from cherries by their suture, waxy bloom on the fruit and a flatter pit (seed).

1.1.3. Apricot

Apricots are grown over a wide range of temperature regimes, ranging from Siberia (-50°C) to North Africa. Apricots belong to the subgenus *Prunophora* Focke, section *Armeniaca* Koch (Layne *et al.*, 1996). There are six species of apricot: *P. brigantina*, *P. armeniaca*, *P. dasycarpa*, *P. mandshurica*, *P. mume* and *P. sibirica*. All apricot species are diploid ($2n = 2x = 16$) and interfertile. *Prunus armeniaca* is the cultivated apricot. Apricots produce perfect, perigynous, white to pink-tinted flowers. Pollen sterility occurs and cultivars may also be self-compatible or self-incompatible. Fruits are freestone or clingstone, round to oval, with glabrous to pubescent fruit skin. Fruit flesh is sweet or sour, more or less aromatic and white, yellow or dark orange in colour (Layne *et al.*, 1996).

1.1.4. Cherries

Cherries originated in the Near East. More than 30 species of cherry have been identified and all are indigenous to Europe and Asia. There are two groups of cultivated cherries. Sweet cherry (*P. avium* L.) is a diploid ($2n = 2x = 16$) and sour cherry (*P. cerasus* L.) is tetraploid ($2n = 4x = 32$). Duke

cherry is a natural hybrid between sour and sweet cherry and produces fruits that are intermediate between these two cherry types. Sweet and sour cherries are classified into subgroups based on skin, flesh and juice colour. The most important sour cherry cultivar in the USA, 'Montmorency', is >100 years old. Cherry flowers are usually white-petalled and single. Cherries can be either self-incompatible or self-compatible.

1.1.5. Almond

Almond is one of the oldest nut crops. It originated in Central Asia and spread to many ancient civilizations in Asia by 2000 BC. It was disseminated to Europe in approx. 350 BC. Almond was once considered to be in a separate genus, *Amygdalus*. The cultivated sweet almond is a diploid ($2n = 2x = 16$) and is now classified as *P. dulcis* (Miller) D.A. Webb with *P. amygdalis* and *P. communis* used as synonyms (Kester and Gradziel, 1996). Several almond cultivars were introduced into California, USA, from the Mediterranean region from approx. 1840 onwards (Kester and Asay, 1975). 'Nonpareil' is the standard California cultivar, and has superior tree and nut characteristics. The cytology of almond is similar to that of other *Prunus* spp. and almond hybridizes with peach (Socias i Company, 1998). In most almond cultivars, flower buds are borne laterally and singly on spurs or shoots. Ripening of almond fruit involves desiccation of the mesocarp to a leathery hull. The pellicle of the kernel also changes colour from white to brown with maturity. The almond nut includes the kernel surrounded by a hard shell. Kernel quality and size, along with shell hardness and shelling percentage, are important factors for cultivar selection (Kester and Asay, 1975).

1.2. Importance

World production of peaches and nectarines is estimated to be approx. 14,787,539 t (FAOSTAT, 2004). The leading producers of peaches and nectarines include China (5,529,366 t), Italy (1,357,352 t), the USA

(1,396,690 t), Spain (1,284,800 t) and Greece (800,000 t). World apricot production is about 2,529,259 t (FAOSTAT, 2004), and the leading producers are Turkey (440,000 t), Iran (284,000 t), Spain (142,300 t), Pakistan (125,000 t) and France (111,000 t). Global production of plums is approx. 10,109,515 t. The leading producers are China (4,234,419 t), Romania (909,648 t), USA (725,290 t), Serbia Montenegro (577,431 t) and Germany (478,730 t). World almond production is approx. 1,679,444 t. The USA (741,440 t), Spain (197,300 t), Syria (139,010 t), Iran (105,000 t) and Italy (91,382 t) are the leading almond producers. California alone produces about 70% of the world's almonds. World cherry production is estimated to be 1,872,436 t (FAOSTAT, 2004), and the leading producers are Iran (220,000 t), the USA (215,000 t), Germany (135,000 t) and Italy (100,518 t).

Stone fruits are consumed fresh, canned, dried or fermented. Peaches, plum, cherries and nectarines provide vitamins, minerals and antioxidants, such as flavonoids, carotenoids and glutathione. Peaches and dried *P. domestica* plums are excellent functional foods for cardiovascular health due to high fibre and potassium content and cholesterol-reducing capacity (Stacewicz-Sapuntzakis *et al.*, 2001; Sun *et al.*, 2002). Ascorbic acid and glutathione in peach are powerful antioxidants able to scavenge the reactive oxygen molecules both in plants and in humans, protect against plant diseases and confer cold tolerance (Kocsy *et al.*, 2001). Antioxidants in the extracts of peach flowers prevent ultraviolet-induced carcinogenesis in guinea pigs and humans (Heo *et al.*, 2001). Wang *et al.* (1999) identified several new antioxidants in dried sour cherries. Almond hulls are a potential source of several dietary antioxidants (Takeoka and Deo, 2003).

1.3. Breeding and genetics

Genetic diversity is narrow for some *Prunus* species (Scorza and Sherman, 1996; Socias i Company, 1998; Scorza *et al.*, 2000). Compared to other fruit species in the

Rosaceae, the peach genome is relatively small (0.61 pg/diploid nucleus or 5.9×10^9 nucleotide pairs) (Arumuganathan and Earle, 1991). The complete sequencing of the peach genome may serve as a model for the genetic improvement of other *Rosaceae*. Several laboratories have produced saturated molecular marker linkage maps for *Prunus* species (Aranzana, 2002). Genetic improvement goals for *Prunus* include: (i) reduced cost of production by increasing productivity; (ii) reduced pest and disease damage (Fig. 18.3.1); and (iii) low-priced, high-quality fruits with long storage life. To achieve these objectives, integration of conventional *Prunus* breeding programmes with genetic mapping and bio-engineering will be important (Scorza, 2001).

1.3.1. Rootstock

Major breeding objectives. Ideally, a *Prunus* rootstock should reduce vigour of the scion; it should be adapted to cold and heat, have resistance to diseases, insects and nematodes and should be compatible with most scion cultivars (Reighard, 2002). Rootstock breeding for plum and apricot is similar to that for peach. Plum rootstocks should have vigour, longevity, resistance to heat, cold and diseases, dwarfing ability, tolerance of drought or flooding, compatibility with scions and ease of propagation (Okie and Weinberger, 1996). Apricot rootstocks should be easy to propagate by seed, with uniform nursery growth and graft compatibility with a broad range of cultivars, and should have cold hardiness and resistance to insects, nematodes, bacteria and virus diseases (Layne *et al.*, 1996). Reduction in tree size, resistance to diseases, such as viruses and *Phytophthora* and *Armillaria* root rots, increased scion precocity, adaptation to a wide range of soils, scion graft compatibility and cold hardiness are important objectives of rootstock breeding for cherries (Brown *et al.*, 1996). Rootstocks for almonds should impart medium to high vigour to support maximum nut yield, possess adaptation to diverse soil types and have insect and disease resistance (Kester and Gradziel, 1996).

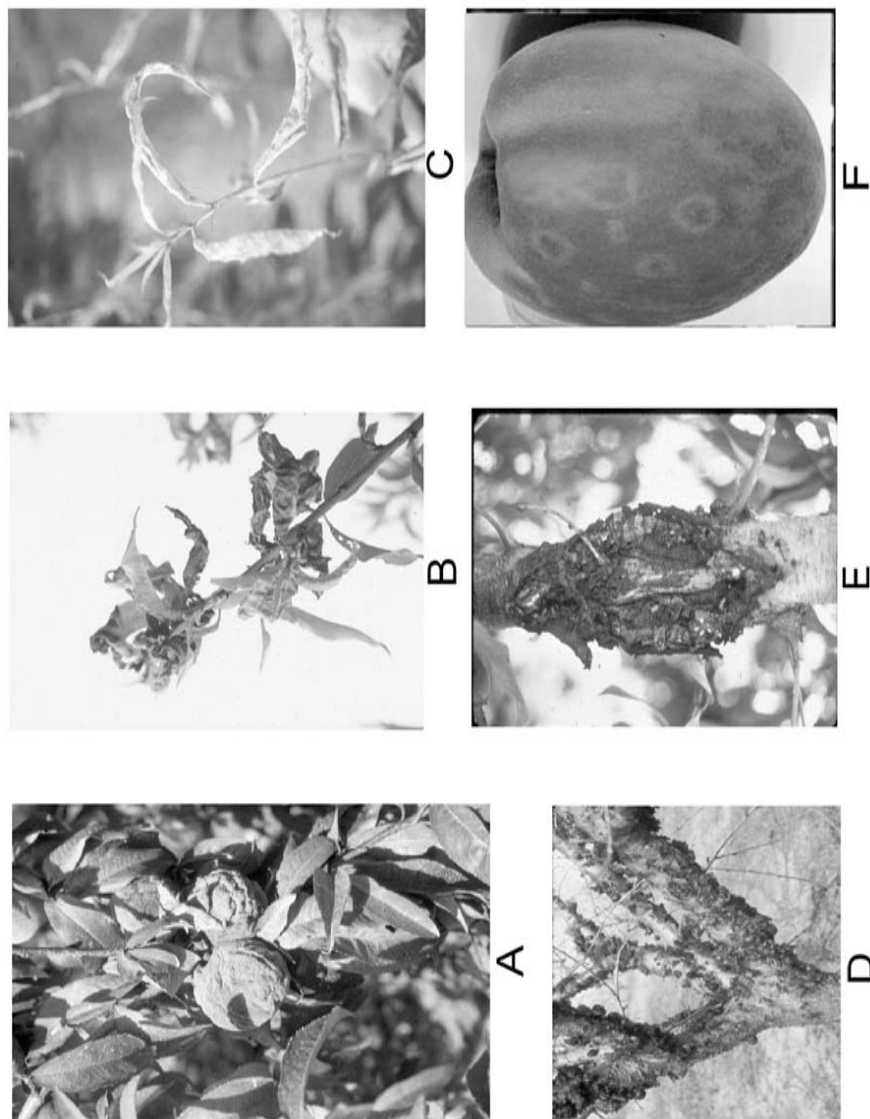


Fig. 18.3.1. Important diseases of peach. (A) Brown rot (*Monilinia fructicola* (Wint.) Honey; (B) leaf curl (*Taphrina deformans* (Berk.) Tul.); (C) powdery mildew (*Sphaerotheca pannosa* (Wallr.:Fr.) Lev.); (D) fungal gummosis (*Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & de Not. = *B. ribes* Gross and Dugg) (courtesy Dr W.R. Okie, USDA, Byron, Georgia, USA); (E) Cytospora canker (*Leucostoma peroonii* (Nits.) Hoehn.) (anamorph: *Cytospora leucostoma* (Pers.) Sacc.) *L. cincta*; (F) plum pox potyvirus (courtesy Dr. Lorne Stobbs, Agriculture and Agri-Food Canada, Vineland Station, Ontario, Canada).

Breeding accomplishments. Rootstocks and scions of most *Prunus* species are compatible with each other, although there are important exceptions.

Peach. Traditionally, rootstocks for peaches have been seedlings. Several nematode-tolerant rootstocks have been released in the USA, Italy, France and Japan (Reighard, 2002). Root-knot nematode (*Meloidogyne* spp.)-resistant rootstocks are recommended for peach where this nematode is endemic. Marker-assisted selection (MAS) has been used to locate certain nematode-resistance genes (Casas *et al.*, 1999; Lecouls *et al.*, 1999). About 26% of 'Redhaven', 'Redglobe' and 'Cresthaven' budded on a nematode-resistant rootstock, 'Nemaguard', succumbed to peach tree short life (PTSL), a disease that involves lesion and dagger nematodes (Beckman *et al.*, 2002; Okie and Scorza 2002). The peach rootstocks, 'Bailey', 'Guardian', 'Rubira' and 'GF 305' are resistant to lesion nematodes (*Pratylenchus vulnus* and *Pratylenchus penetrans*) and dagger nematodes (*Xiphinema americanum*) (Reighard, 2002). Peach rootstocks are generally unsuitable for poorly drained, heavy clay or calcareous soils. Breeders in France have developed several rootstocks adapted to calcareous soil (Reighard, 2002). Cold-hardy rootstocks have been released in Canada (Layne, 1987), Russia and the Czech Republic (Reighard, 2002). A comparison of 'Redhaven' peach scion on nine rootstocks and at 16 locations known for heavy soils and occurrence of winter cold damage showed greatest yield on rootstocks 'GF 677', 'Halford', 'Bailey' and 'Siberian C' (Perry *et al.*, 2000). There is no satisfactory rootstock for vigour and size control of peach. Several rootstocks have been released in Europe and the USA, but none of them significantly reduce vigour in peach (Scorza and Sherman, 1996; Reighard, 2002).

Plum. Ramming and Cociu (1991) have described the genetic resources for plum. The leading rootstocks are 'Myrobalan', *P. domestica*, *P. cerasifera*, 'Marianna' and peach (Okie and Weinberger, 1996). 'Penta' and 'Tetra' (*P. domestica*) rootstocks that

have originated in Italian breeding programmes are mostly compatible with peach, plum, apricot and nectarine (Nicotra and Moser, 2001). Selections and hybrids have been made in East Malling, UK, and Institut National Recherche Agronomique (INRA) in Bordeaux, France, from traditional rootstocks (Okie and Weinberger, 1996). New clonal rootstocks include 'GF 43' and 'Pixy' from Italy (Okie and Weinberger, 1996) and 'Citation' from California, USA (Okie and Weinberger, 1996). Peach $\times$ plum hybrids such as 'Yumir' and 'Ferciana' have also been tested as rootstocks (Okie and Weinberger, 1996). Japanese plums are routinely grown on peach rootstocks 'Nemaguard' and 'Lovell' (Okie and Weinberger, 1996).

Apricot. Rootstock breeding for apricot has been rather limited. Seedlings of several *Prunus* species and their hybrids have been used as apricot rootstocks (Layne *et al.*, 1996).

Almond. Almond $\times$ peach hybrids show outstanding characteristics as rootstock for almond (Feliipe *et al.*, 1998a,b). All self-fruitful almonds tested have grown vigorously and produced consistently higher yields when grafted on the peach $\times$ almond hybrid rootstock 'GF 677', compared to peach seedling rootstock 'GF 3054' (Godini and Palasciano, 1998).

1.3.2. Scions

Major breeding objectives. For all *Prunus* fruit species, high productivity, improved fruit quality and resistance to pests and diseases are major breeding objectives (Scorza and Sherman, 1996). Cold hardiness, late blooming to escape spring frosts, extension of season of maturity and delayed softening of ripe fruits are also important (Callahan *et al.*, 1991).

Peach. Greater diversity of fruit types, improved fruit storage life and modified tree architecture are also being pursued by peach breeding programmes worldwide (Marin and Sowers, 2000; Byrne, 2002). Genetic manipu-

lation of scion growth habit to control tree size and the breeding of narrow peach leaf morphology to improve photosynthesis are among the objectives of US Department of Agriculture (USDA) breeding programmes (Scorza *et al.*, 1989a, 2000, 2001a; Okie and Scorza, 2002). Peach cultivars with a long period of winter dormancy are especially sought (Layne *et al.*, 1996).

Apricot. Climatic adaptation, resistance to diseases, extension of the ripening season and increased fruit storage life are primary objectives of apricot breeding programmes worldwide. Resistance to 'apoplexy', a term used to describe sudden wilting and death of a tree or part of a tree, and good pomological fruit characteristics, e.g. large fruit size, freestone, firm flesh and resistance to skin cracking, are additional objectives of apricot breeding (Layne *et al.*, 1996).

Plum. Attractive fruit appearance, large size, firmness, acceptable flavour and texture and resistance to diseases are the breeding objectives for plum (Okie and Weinberger, 1996). In addition, early ripening, high productivity and processing quality for drying and brandy making are important (Okie and Weinberger, 1996). For Japanese plums, dark blue colour, firmness for shipping, low chilling requirements and disease resistance are important (Okie and Weinberger, 1996). In Europe, major concerns include better adaptation to soil and climate and resistance to sharka disease (plum pox virus (PPV)) and apoplexy (Okie and Weinberger, 1996).

Cherry. Compact growth habit, late flowering, resistance to fruit cracking and *Pseudomonas*, precocity in bearing, large firm fruits, earlier and later ripening, high yield, mechanical harvesting ability and good shipping quality are principal breeding objectives for cherry (Brown *et al.*, 1996). Important goals in sour cherry breeding include the development of cultivars with improved fruit quality, delayed bloom time (to avoid spring freezes) and a greater range of ripening dates (Iezzoni *et al.*, 1991; Brown *et al.*, 1996).

Almond. Self-compatibility and productivity are primary objectives of almond breeding. In addition, blooming date to mitigate adverse weather conditions, resistance to pests and diseases and improved kernel quality are other modern breeding objectives (Kester and Gradziel, 1996; Sicias i Company, 1998). The absence of severe crossing barriers permits almond to hybridize with several related *Prunus* spp. to expand germplasm available to breeders (Gradziel *et al.*, 2001).

Breeding accomplishments.

Peach. At least 60–70 peach cultivars are released annually worldwide (Byrne, 2002). Most varieties are released based on improved fruit characteristics including extension of the fruiting season, increased size, red skin colour, firmness, improved flavour, longer storage life and increased resistance to biotic and abiotic stress. Current world peach production depends on the use of scions with a vigorous spreading canopy. Compared to apple, peach yield is low (Scorza *et al.*, 2000). Severe pruning is necessary to promote new fruiting wood. Breeding programmes are approaching this problem by developing alternative growth habits and spur-type peach cultivars, which may allow increased productivity in high-density plantings (Scorza, 1984; Scorza and Sherman, 1996; Marin and Sowers, 2000; Scorza *et al.*, 2000). Breeding programmes in the USA and Italy have produced peach trees with columnar (pillar) growth habits and high-quality fruit (Scorza, *et al.*, 1989a, 2000). Peaches with low chilling requirements and early fruiting cultivars have been released from Florida and California, USA (Scorza and Sherman, 1996; Ramming and Tanner, 1987a,b).

Plum. Modern breeding programmes have produced many vigorous high-quality plums suitable for the dessert market (Scorza and Fogle, 1999). Several European plum cultivars were released from the New York Experimental Station, Geneva, New York, USA. 'Stanley', which was released in 1926, is still popular worldwide (Ramming and Cociu, 1991). Unlike European plums, most Japanese plums have been bred recently (Okie and Weinberger, 1996). More than 200

Japanese plum cultivars are under commercial production in California alone (Okie and Weinberger, 1996).

Apricot. Cold-hardy, drought- and heat-resistant apricot cultivars have been developed in Russia (Layne *et al.*, 1996). The Harrow Research Station, Ontario, Canada, has released several apricot cultivars with resistance to bacterial leaf spot, brown rot (*Monilinia fructicola*) and canker (*Leucostoma* spp.) (Layne *et al.*, 1996). Cultivars that have been developed from the Central Asian and Irano-Caucasian group of apricots are high in soluble solid content (> 20%) and are used for the dried apricot industry (Layne *et al.*, 1996).

Cherry. The development of several disease-resistant, self-fertile cherry cultivars in Canada has represented an exciting advance in sweet cherry breeding (Brown *et al.*, 1996). While sour cherry cultivar development, e.g. the popular landrace cultivar 'Pandy' in Europe and 'Plodorodnaya Michurina' in Russia, has been noteworthy (Brown *et al.*, 1996), 'Montmorency', a 100-year-old landrace sour cherry, is still the most popular sour cherry cultivar in the USA.

Almond. Ten almond cultivars from California, USA, and nine cultivars from the Yalta region of Russia were released between 1932 and 1996 for commercial production (Kester and Gradziel, 1996), but 'Nonpareil' and 'Mission' are still the dominant cultivars in California, USA.

2. Molecular Genetics

2.1. Gene cloning and functional genomics

A worldwide consortium of laboratories is attempting to build genomic resources for peach to be used as a model for the identification, cloning and characterization of genes in *Prunus* (Fig. 18.3.1) (Abbott *et al.*, 2002; *Prunus* Genome Database, 2002).

Since the fruit is the ultimate product of stone fruits, ripening-related genes are widely studied. Genes associated with gametophytic self-incompatibility, i.e. the

S-ribonuclease (RNase) genes, are also of interest to many researchers. Recently, antioxidant genes, pathogenesis-related (PR) genes and genes that encode human allergens have come under study. Since almond is cultivated for nuts, storage protein and lipid genes have been targeted.

Many genes are involved in ripening and softening of fruits. Rapid softening of ripe peaches is a major problem in storage, transport and marketing (Callahan *et al.*, 1991; Scorza, 1991, 2001). Cell wall-loosening hydrolases, including glucanases, cellulases and pectic enzymes are involved in peach fruit softening (Bonghi *et al.*, 1998). Genes controlling the cell wall pectic enzyme polygalacturonase (PG) have been isolated from several species (Hadfield and Bennett, 1998). Three distinct activities of PG have been identified in peach fruits; two of them act as an exo-PG, while one is an endo-PG. Exo-PG activity in peach is often ripening regulated (Downs *et al.*, 1992). Endo-PG is more strongly associated with freestone peaches and a genetic linkage between endo-PG and freestone has been identified (Lester *et al.*, 1996). Endo- β -1,4-glucanases (ppEG1) accumulate in peach during ethylene-associated fruit and leaf abscission (Trainotti *et al.*, 1997). They also appear to be involved in early fruit development and the early phases of fruit softening. Softening is strongly inhibited in 'Redhaven' peaches treated with 2,5-norbornadiene, which inhibits endoglucanase gene transcripts (Bonghi *et al.*, 1998). Peach endoglucanase-1 (ppEG1) gene has seven exons and a putative signal peptide and it shows 76% homology with ripening-related avocado glucanase (Trainotti *et al.*, 1997). Expansin, a ripening-related gene, increases prior to the development of cold storage-induced mealiness in peach (Obenland *et al.*, 2001). A low-molecular-weight heat-shock protein is expressed during fruit ripening in peach (Callahan and Cohen, 1994). A defence-related β -glucosidase gene is expressed in ripening sweet cherries (Gerardi *et al.*, 2001), and a polyphenol-oxidase gene is expressed in leaves and mature fruits of apricot and is later turned off during fruit ripening (Chevalier *et al.*, 1999). The polyphenol-oxidase gene

appears to be structurally and enzymatically similar among *Rosaceae* fruit species (Haruta *et al.*, 1999).

The ethylene climacteric in peaches coincides with or follows softening. Two ethylene biosynthetic enzyme families, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase, accompany ripening in peach (Callahan *et al.*, 1993a,b,c). A fruit-specific, transcriptionally regulated 36 kDa ACC oxidase protein has been found in apricot 'Bergeon' (Mbeguie-A-Mbenguie *et al.*, 1999). Exposure of green fruits of Japanese apricot (*P. mume*) to exogenous ethylene induces both ACC synthase and ACC oxidase, but wounding of pericarp tissue induces only ACC synthase gene expression (Mita *et al.*, 1999). More ethylene biosynthetic enzymes accumulate in the fast softening peach cultivar 'Bailey', compared to the slow softening cultivar 'Suncrest' (Callahan *et al.*, 1992). Fruits of the ethylene mutant peach cultivar 'Yumyeong' produce little or no ethylene and remain firm during storage (Haji *et al.*, 2001). This cultivar is popularly called 'stony hard' peach. The stony hard gene is recessive, and increases fruit firmness and shelf-life (Goffreda, 1992). Ruperti *et al.* (2001) compared the expression of two peach ACC oxidases and found that they were differentially expressed in flowers, young fruits, seedlings and expanded leaves. One gene, *PP-ACO2*, is expressed only in fruit and is unaffected by propylene treatments, while the other, *PP-ACO1*, strongly accumulates in senescing leaves, abscising fruit and ripe mesocarp, and is enhanced by propylene treatments. Vizzotto *et al.* (2002) delayed the onset of fruit softening and fruit ethylene emission in 'Redhaven' by applying the ethylene inhibitor aminoethoxyvinylglycine (AVG). To study the role of peach ACC oxidase in fruit softening, transgenic plum trees containing antisense peach ACC oxidase have been regenerated and are now being evaluated in the field (A. Callahan, USDA, Kearneysville, personal communication). Two peach ethylene receptor genes, *Pp-ETR1* and *Pp-ERS1*, homologous to *Arabidopsis ETR1* and *ESR1*, have been isolated (Bonghi *et al.*, 2002). The level of *Pp-ETR1* transcripts remains unchanged throughout ripening,

whereas *Pp-ERS1* increases in parallel with the ethylene climacteric. Treatment of fruits with the ethylene action inhibitor 1-methylcyclopropane transiently reduced transcripts of both receptor genes and also of ethylene evolution. An orthologue of *Arabidopsis ETR1* (*PpETR1*) isolated from peach displays an unusual transcript processing property that is different from that of *Arabidopsis* and tomato *ETR1* (Bassett *et al.*, 2002). Ethylene-responsive genes from peach have been isolated from cells of the abscission zone evoked by treatment with the ethylene analogue propylene. Proteins of these genes, termed *PpAz*, showed homology to PR thau-matin-like proteins and plant and fungal β -D-xylosidases. The transcripts of three PR-like genes *PpAz8*, *PpAz44*, *PpAz152* accumulated preferentially and preventively in the fruit and leaf abscission zones through an ethylene-dependent pathway (Ruperti *et al.*, 2002).

Gametophytic self- and cross-incompatibility in *Prunus* is controlled by an S locus consisting of the S-RNase and an unidentified 'pollen S gene'. In sweet cherry, cDNA sequences of six S-RNase (S1 to S6) alleles have been isolated (Tao *et al.*, 1999). A novel S-RNase, S-2 (X), found in a Japanese sweet cherry cultivar, is different from the six known S-RNase genes (Yamane *et al.*, 2000). Molecular analyses of three T2/RNase-type RNase genes in almond showed four conserved regions (C1, C2, C3 and C5) and one *Rosaceae*-specific (RC4) conserved region (Ushijima *et al.*, 1998). The T2/RNase genes in Japanese apricot (*P. mume*) also displayed similar conserved regions (Yaegaki *et al.*, 2001). A distinct class of S-like RNase (RNase PD2) gene is expressed in petals, pistils and leaves of almond (Ma and Oliveira, 2000), and a non-pistil-specific S-RNase-like gene (PD1) is reportedly present in a self-incompatible almond cultivar (Van Nerum *et al.*, 2000). Japanese apricot (*P. mume* Shieb. et Zucc.) exhibits gametophytic self-incompatibility, but the self-compatible Japanese apricot cultivars have in common the S-RNase gene designated as S^f-RNase (Tao *et al.*, 2002). This gene was inherited from either the seed or pollen parent as a pistil S allele in a non-functional S haplotype.

Abiotic and biotic stress-related genes have been identified in stone fruits and nuts. Both transcripts and proteins of a seasonally expressing, stress-induced dehydrin gene (*ppdhn1*) accumulate more in cold-tolerant deciduous peach tree cambium than in the less cold-tolerant 'Evergreen' cultivar (Artlip *et al.*, 1997; Wisniewski *et al.*, 1999). Two complementary DNA (cDNA) clones encoding manganese superoxide dismutase (MnSOD) were cloned from peach, which showed 94% homology to an MnSOD from *Nicotiana plumbaginifolia* (Bagnoli *et al.*, 2002). These genes contain a 24-amino acid transit peptide typical of those used to target proteins to the mitochondria. The PR defence genes β -1,3-glucanases have been induced in peach following exposure to culture filtrates of *Xanthomonas campestris* pv. *pruni* (Thimmapuram *et al.*, 2001). Flavoprotein mandelonitrile (MDL) lyase plays a key role in cyanogenesis and defence in stone fruits. A family of eight MDL genes has been found in black cherry (Hu and Poulton, 1999). In almond, a flower-specific gene for a flavonoid biosynthetic pathway enzyme has binding sites for S-adenosyl-L-methionine (SAM) (Suelves and Puigdomenech, 1998).

Two chlorophyll *a/b*-binding (*cab*) nuclear genes, *Lhcb2*Pp1* and *Lhcb2*Pp1*, associated with photosystem II, are expressed in green leaves of peach (Bassett *et al.*, 1998; Chung *et al.*, 1998). Four potential polyadenylation sites are found in the *cab* gene *Lhcb1Pp1* in peach leaves, whereas in the stem there are only three sites. Since the *Lhcb2*Pp1* gene expresses mostly in green tissues, the fully ripe peach fruits may not accumulate *cab* gene products (Callahan *et al.*, 1993c). Accordingly, the peach *cab* promoter has been cloned and is now used to drive green tissue-specific expressions of foreign genes in *Prunus* (C. Bassett, USDA, Kearneysville, USA, personal communication). Two seed-specific genes that code for prunin and oleosin proteins, which are involved in early seed development, are localized in the cotyledons of almond cultivar 'Texas' (García-Mas *et al.*, 1995). Acosta *et al.* (1999) purified a major almond seed protein, which can be used to detect the presence of almond in food using competitive enzyme-linked

immunosorbent assay (ELISA). A lipoxynase (9-LOX) is expressed during early seed development in almond. This gene is similar to potato tuber and tomato fruit lipoxynases (Mita *et al.*, 2001).

Peaches contain some compounds which cause food allergies, and a family of 9 kDa lipid transfer proteins (LTPs) has been identified as a major allergen of peach (Malet *et al.*, 1988; Pastorello *et al.*, 1999) and other rosaceous fruits (Asero *et al.*, 2001). LTPs are abundant in nature and transfer lipids from donor to acceptor membranes. Isoforms of LTPs are multifunction proteins, which have a role in plant defence, abiotic stress and embryogenesis (Botton *et al.*, 2002). Amino acid sequences of apricot LTPs have 91% and 94% similarity to peach and almond LTPs, respectively (Conti *et al.*, 2001). LTPs bind with immunoglobulin E and cause type I allergic reactions in humans (Sanchez-Monge *et al.*, 1999; Hoffman-Sommergruber, 2000; Asero *et al.*, 2001). Two LTP genes, *pp-LTP1* and *pp-LTP2*, are found in peach. A transcript of *pp-LTP1* accumulates only in the epicarp of ripening peach fruits and does not accumulate in vegetative tissues or flowers, whereas *pp-LTP2* expresses only in the ovary. The *pp-LTP1* protein is concentrated in peach skin, and is the major allergen in peach (Botton *et al.*, 2002). PR thaumatin-like proteins, Pru a1 and Pru a2, are major allergens in cherry (Scheurer *et al.*, 1997; Inschlag *et al.*, 1998). A thaumatin-like gene is abundantly expressed when cherry fruits change colour from yellow to red (Fils-Lycaon *et al.*, 1996).

2.2. Marker-assisted selection

Molecular markers are valuable tools to locate the economically important *Prunus* genes using linkage maps (Fig. 18.3.1). Undesirable plants can be eliminated from progeny populations using MAS as early as the seedling stage (Scorza and Sherman, 1996; Scorza, 2001). Restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLPs) are widely used to generate linkage maps. In addition, simple sequence repeat

(SSR), intermicrosatellite amplification (IMA) and bulked segregant analysis (BSA) are used to study traits and to identify cultivars. Linkage maps are most advanced for peach (Fig. 18.3.1; Chaparro *et al.*, 1994; Dirlewanger and Bodo, 1994; Rajapakse *et al.*, 1995; Abbott *et al.*, 1998; Dirlewanger *et al.*, 1998c; Sosinski *et al.*, 1998; Aranzana *et al.*, 2002). Linkage maps are also available for almond (Viruel *et al.*, 1995), sweet cherry (Stockinger *et al.*, 1996), sweet cherry $\times$ *P. incisa* and sweet cherry $\times$ *P. nipponica* (Boskovic *et al.*, 1997), peach $\times$ almond (Foolad *et al.*, 1995; Joobeur *et al.*, 1998) and sour cherry (Wang *et al.*, 1998). Eight linkage groups have been identified for peach and almond (Fig. 18.3.1), whereas ten linkage groups in sweet cherry and 18 linkage groups in the polyploid sour cherry have been identified. Using RFLP markers with almond $\times$ peach crosses, Joobeur *et al.* (1998) constructed a general *Prunus* linkage map with eight linkage groups to screen all *Prunus* species as well as apple. Recently Bliss *et al.* (2002) expanded the genetic linkage map of *Prunus* based on the almond $\times$ peach cross.

Several linkages and markers have been identified in peach. Pillar (*Br*), double flowers (*Dl*) and flesh colour loci are linked (Rajapakse *et al.*, 1995). In Japanese peach cultivars, the brachytic dwarf (*dw*) and narrow leaf (*nl*) loci are located in the same linkage group as red leaf (*Gr*) at a distance of 65 cM. The narrow leaf (*nl*) character cosegregated with *dw* locus (Shimada *et al.*, 2000). Three markers in peach are closely linked to the freestone (*F*) trait, the locus controlling flesh/stone adherence (Sosinski *et al.*, 1998). Linkage has also been found between *Sphaerotheca pannosa* resistance, flesh adhesion and certain molecular markers (Quarta *et al.*, 1998). Linkage relationships between molecular markers and quantitative trait locus (QTL) analyses have been reported for fruit quality, powdery mildew resistance, internode length, blooming time, ripening time, skin colour, saucer fruit gene, peach leaf curl and *S. pannosa* resistance and soluble solid content (Dirlewanger *et al.*, 1996, 1998a, 1999; Quarta *et al.*, 1998; Viruel *et al.*, 1998; Wang, Y. *et al.*, 2000; Verde *et al.*, 2001;

Bellini *et al.*, 2002; Etienne *et al.*, 2002; Guo *et al.*, 2002; Lecouls *et al.*, 2002). Epistasis has been observed between QTLs for most of the characters analysed (Dirlewanger *et al.*, 1998b).

Sour cherry is a tetraploid and exhibits the disomic inheritance of an allotetraploid. These traits add to the complexity of linkage map construction. Single-dose restriction fragment (SDRF) low-density RFLP linkage maps have been used to identify tetraploid sour cherry cultivars (Wang *et al.*, 1998). Four of the sour cherry linkage groups may be homologous with four of the eight genetic linkage groups identified in peach and almond. Wang *et al.* (1998) used linkage maps and 17 linkage groups to estimate locations and effects of QTLs affecting various flower and fruit traits. Polymorphism was studied in crosses of two almonds using 12 isozyme loci and RFLP and RAPD primers (Arulsekhar *et al.*, 1989; Arus *et al.*, 1994; Viruel *et al.*, 1995). Eight linkage groups were found, but none of the loci examined were unlinked. A second-generation linkage map, generated using progeny of the same two almond cultivars, increased the map size by 5% (Joobeur *et al.*, 2000; Fig. 18.3.1). Several genes related to drought tolerance were identified by employing a 'differential expression cDNA-AFLP technique' with *in vitro*-cultured almond plantlets subjected to desiccation or abscisic acid (ABA) treatment (Campalans and Pages, 2001). Ballester *et al.* (2001) mapped a major gene for delayed blooming time in almond.

To classify apricot cultivars, Takeda *et al.* (1998) used RAPD and almond RFLP genomic and cDNA markers. By fingerprinting with RAPD primers, Wooley *et al.* (2000) showed that almond cultivars originating from Europe or the Middle East clustered into a different group from those originating from California. AFLP and microsatellites are more powerful fingerprinting tools to identify the origin of *Prunus* species (Cervera *et al.*, 2000; Struss *et al.*, 2001; Dirlewanger *et al.*, 2002a). SSR markers are highly polymorphic, easily reproducible co-dominant markers (Wang *et al.*, 2002). SSR markers can identify closely related peach cultivars (Badenes *et al.*, 2002). Georgi *et al.* (2002) developed bacterial

artificial chromosome (BAC) libraries to identify the abundance and local distribution of SSRs in peach. They found that microsatellite loci are not randomly distributed within gene-containing regions of the peach genome. Seventeen AC/GT- and AG/CT-rich microsatellite loci have been found in genomic libraries of 'Redhaven' peach (Cipriani *et al.*, 1999). Ten of these loci showed Mendelian inheritance and 15 microsatellites showed polymorphism with two to four alleles each in ten genotypes, with cross-transportability to other *Prunus* species, as well as to apple. Microsatellites gave high heterozygosity in all loci and confirmed parenthood of most cultivars, but were unable to resolve the sport mutants, e.g. 'Compact Redhaven', from the 'Redhaven' parent (Testolin *et al.*, 2000). By developing a high-throughput approach to searching the peach genome for SSRs linked to traits of interest using an AFLP genetic linkage map and a peach BAC library (Wang, Y. *et al.*, 2000), Wang *et al.* (2002) identified SSR loci tightly linked to simply inherited traits in peach, e.g. root knot nematode resistance and the control of the evergrowing trait. Microsatellite primer pairs developed in peach have been successfully used to produce polymorphic amplification patterns for SSR loci to identify 76 sweet cherry genotypes (Wunsch and Hormaza, 2002), demonstrating the cross-species transferability of microsatellite sequences. Similarly, Downey and Iezzoni (2000) used a chloroplast DNA marker from sour cherry and eight nuclear SSR markers tested in peach, sour cherry and sweet cherry to study the genetic diversity of black cherry (*P. serotina*) and to determine the ancestral origin of the Mexican 'Capulin'. Very high cross-species transportability of microsatellites from peach to other *Prunus* spp., as well as to other rosaceous (apple and strawberry) and non-rosaceous (chestnut, grapes and walnut) species, makes microsatellites powerful markers for synteny analysis in *Prunus* (Dirlewanger *et al.*, 2002b). Twenty-four single locus SSRs, highly polymorphic in peach and each falling within 24 evenly spaced 25 cM regions covering the whole *Prunus* genome, are proposed as a 'genotyping set' as a reference for finger-

printing, pedigree and genetic analysis of *Prunus* species (Aranzana *et al.*, 2002). The resulting *Prunus* genetic map (Fig. 18.1.1) contains a total of 342 SSR markers covering a total distance of 522 cM. SSRs are placed in all the eight linkage groups of this map, and the distribution is relatively even, providing a genome-wide coverage with average density of 5.2 cM/SSR (Aranzana *et al.*, 2002).

Self-incompatibility and male sterility are two traits that limit apricot improvement and markers can be used for early determination of these phenotypes (Badenes *et al.*, 2000). No markers have been linked to S alleles, but three markers are linked to male-fertility and two markers are linked to a self-compatible (*Sc*) allele. All self-incompatible cultivars of Japanese apricot (*P. mume*) have a common S-RNase allele, which can be used as a marker for self-compatibility (Tao *et al.*, 2000). Gametophytic self-incompatibility in almond is controlled by a single multi-allelic locus (S locus). In styles, the products of S alleles are S-RNases. Cultivated almonds in California have four predominant S alleles (S-a, S-b, S-c, S-d) (Tamura *et al.*, 2000). An almond cultivar, 'Jeffries', a somaclonal mutant of 'Nonpareil', has a dysfunctional S-c haplotype in the pistil and pollen (Ushijima *et al.*, 2001). Similar markers have been identified in cherry using polymerase chain reaction (PCR) primers (Sonneveld *et al.*, 2001). With sweet cherry, the RFLP closest to the bloom time QTL has been detected by a cDNA whose partial amino acid sequence is 81% identical to that of a Japanese pear stylar RNase (Wang, H.B. *et al.*, 2000b). Esterase gene, *Est-5*, is linked to stylar RNase (incompatibility locus S) in sweet cherries (Boskovic *et al.*, 2000). About a fifth of 50 cultivars genotyped for *Est-5* are heterozygous. The heterozygotes could act as 'testers' to identify eight of the 11 known S alleles in cherry cultivars of unknown genotype.

MAS is employed for *Prunus* rootstock breeding for nematode resistance (Lu *et al.*, 1998, 1999). Several cultivars of Myrobalan plum are highly resistant to all root knot nematodes, *Meloidogyne* spp. Each cultivar bears a single major dominant gene that controls wide-spectrum root knot nematode resistance. Using RAPD and sequence-char-

acterized amplified region (SCAR) markers and BSA analysis, two dominant markers have been identified in selected resistant *Prunus* rootstocks (Lecouls *et al.*, 1999). Blenda *et al.* (2002) made crosses between BY520-9 peach, a ring nematode-tolerant peach, with 'Nemaguard', a susceptible rootstock, and identified a resistance marker for ring nematode, which is associated with the PTSL disease syndrome.

3. Micropropagation

Micropropagation of *Prunus* is primarily used for virus elimination and rootstock propagation. Micropropagation of cherry, plum and peach have been reviewed (Marin and Gella, 1991; Druart, 1992; Scorza and Hammerschlag, 1992).

Micropropagation offers the potential for mass production of own-rooted peach, which may be useful as rootstocks for virus indexing, and can accelerate screening for disease resistance (Reeves and Couvillon, 1992). Shoot tips and nodal cuttings are the usual explants. The large-scale commercial micropropagation of virus-free plants of the peach rootstocks 'Istara', 'GF677', 'Penta', 'Tetra', 'MrS', 'Fire Cadman', 'Barrier1', 'Gensia' and 'Julior' has been reported (Battistini and De Paoli, 2002). Benzyladenine (BA) is the cytokinin most often used for multiple shoot proliferation in peach, although its optimum concentration varies with genotype. 'Dixie Red' and 'Springtime' require 3.5–5 μM BA, whereas 'Lovell' and 'Nemaguard' shoots proliferate best on semi-solid medium containing 26.7 μM BA and 0.04 μM indolebutyric acid (IBA) (Parfitt and Almehdi, 1986). Modified Murashige and Skoog (1962) (MS) medium supplemented with 9 μM BA has been used to micropropagate eight peach scions and 'Nemaguard' rootstock (Reeves and Couvillon, 1992). 'Sunhigh' and 'Compact Redhaven' grew better in liquid medium; however, 'Nemaguard' failed to grow in liquid medium. Shoot tip cultures have been successfully used to micropropagate almond $\times$ peach hybrid clones, i.e. 'GF677' (Kester and Gradziel, 1996), 'Hansen 536' and

'Hansen 2168' (Kester and Asay, 1975). Apricot cultivars have been micropropagated on semi-solid modified woody plant medium (WPM) (Lloyd and McCown, 1981) supplemented with 2.25 μM BA. About 92.8% of the shoots rooted in medium containing 10.8 μM naphthaleneacetic acid (NAA) but the number of roots per shoot was higher in medium supplemented with 22.5 μM IBA (Perez-Tornero *et al.*, 2000a).

The use of zygotic embryo culture as a tool to rescue embryos from abortion and for micropropagation has been reviewed (Ramming, 1990). Early-maturing peach and nectarine seedlings have been grown from 1–5 mm long immature zygotic embryos (Emershad and Ramming, 1994). To accelerate the breeding process in sweet cherry, Hormaza (1999) combined *in vitro* embryo culture and MAS.

Vegetative propagation of mature plants is difficult compared to that of their juvenile counterparts. Micropropagation may circumvent the effects of ageing or maturation, or both (Hammatt and Grant, 1993, 1997). Micropropagated plum trees produced cuttings with improved rooting 9 years after establishment, probably due to partial rejuvenation of mature tissues (Howard *et al.*, 1989).

4. Micrografting to Eliminate Virus

Prerequisites for successful *in vitro* virus-indexing are identifiable indicators of virus infection. *In vitro* meristem culture can then be used to produce disease-indexed plants, e.g. cherry (Buzkan *et al.*, 1997). Zilkah *et al.* (1995) attempted to recover plants free of *Prunus* necrotic ringspot virus (PNRSV) using micropropagated shoots of *P. serratula* 'Shirofugen'. Shoot cultures of the infected peach 'Elberta' and the cherry '40E50' were used as rootstocks on to which 'Shirofugen' shoots were micrografted. The typical symptomatic necrosis of PNRSV developed on 'Shirofugen' shoots. An efficient micrografting method has been developed for the almond 'Achak' to produce virus-indexed scions for almond orchards in Tunisia (Ghorbel *et al.*, 1998).

5. Somatic Cell Genetics

5.1. Regeneration

5.1.1. Somatic embryogenesis

Although induction of somatic embryogenesis was reported for several *Prunus* spp., conversion of these somatic embryos into plants is far from routine (Srinivasan and Scorza, 1999). Druart (1999) has reviewed the current status of somatic embryogenesis in *Prunus*.

Peach and nectarines

Induction. Raj Bhansali *et al.* (1990) induced somatic embryos from 1–3 mm long immature zygotic embryos of peaches and nectarines. To induce embryogenic cultures, the zygotic embryos were cultured on semi-solid MS basal medium containing 500 mg/l each of glutamine, myo-inositol and casein hydrolysate, 22.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 9 μM kinetin and 9 μM BA. The explants were initially incubated for 10 days in darkness and then exposed to continuous light for 20 days at 23°C.

Maintenance. Raj Bhansali *et al.* (1990) induced secondary somatic embryogenesis from somatic embryos on semi-solid medium with 5 μM 2,4-D. A few mature somatic embryos developed a normal shoot/root axis on semi-solid MS medium containing 9 μM BA and 500 mg/l casein hydrolysate. Embryogenic suspension cultures have been established from secondary embryos by culturing them in liquid MS medium containing 1000 mg/l myo-inositol, 500 mg/l glutamine, 500 mg/l casein hydrolysate, 5 μM 2,4-D, 2 μM BA and 2 μM kinetin (Raj Bhansali *et al.*, 1991). Heart-shaped embryos develop in suspension cultures if the above medium contains 1000 mg/l calcium nitrate and 6% sucrose. Embryogenic cell suspensions have been maintained for 10 months by subculturing every 2 weeks into a fresh medium.

Development/maturation. For somatic embryo development, embryogenic cultures are transferred on to semi-solid MS basal

medium with 500 mg/l each of glutamine, myo-inositol and casein hydrolysate, 976 mg/l 2-(*N*-morpholino)ethane sulphonic acid (MES) and 2.5 g/l activated charcoal without growth regulators. Somatic embryos develop on this medium after three to five transfers at 3-week intervals, although most somatic embryos fail to germinate. Scorza *et al.* (1990a) maintained habituated embryogenic cultures for 4 years on semi-solid MS medium. Production of somatic embryos from these cultures is not affected by NAA or BA, and they seldom germinate.

Germination. Plants develop sporadically from mature somatic embryos on semi-solid MS medium supplemented with 0.45 μM BA, 0.54 μM NAA and 0.03% activated charcoal (Raj Bhansali *et al.*, 1990). Somatic embryos from 18-month-old and 4-year-old habituated embryogenic peach cultures occasionally germinate on Driver and Kuniyuki (1984) walnut medium supplemented with 0.1 μM IBA and 5 μM BA (Scorza *et al.*, 1990a).

Cherry

Induction. Embryogenic cultures of wild sweet cherry (*P. avium*) have been induced from cotyledons of immature zygotic embryos (2.5 to 4.5 mm long) after 10 days in darkness at 25°C on semi-solid MS medium containing Morel vitamins (Morel and Wetmore, 1951), 20 g/l sucrose, 500 mg/l casein hydrolysate, 250 mg/l glutamine, 2 mg/l glycine, 18.1 μM 2,4-D and 9.3 μM kinetin. Mandegaran *et al.* (1999) induced embryogenic cultures from roots of 'Colt' on semi-solid MS medium supplemented with 1.5 μM BA and 15 μM 2,4-D. Secondary embryos were produced from these somatic embryos when transferred to a medium containing 1.5 μM BA, 10 μM IBA and 5 μM 2,4-D. Embryogenic cultures have been induced from immature cotyledons from zygotic embryos of open-pollinated fruit of three sour cherry (*P. cerasus*) cultivars on semi-solid MS medium supplemented with 2 to 22.5 μM 2,4-D and 4.5 μM kinetin (Tang *et al.*, 2000).

Maintenance. The embryogenic line Q36 was identified by De March *et al.* (1993) and

was maintained on semi-solid MS medium containing 0.4 μM BA, 0.5 μM kinetin and 0.5 or 2.5 μM IBA for six subcultures over a period of 3 years (Garin *et al.*, 1997).

Development/maturation. Somatic embryos of sweet cherry develop from embryogenic cultures following their transfer to hormone-free medium, but do not develop normally (De March *et al.*, 1993). Shoots developed from 75% of 'Colt' cherry somatic embryos on semi-solid medium containing 2 μM BA and 3 μM gibberellic acid (GA_3), although roots did not develop (Mandegaran *et al.*, 1999). A combination of 10 μM abscisic acid (ABA) and 88 mM maltose can improve sweet cherry somatic embryo development (Reidiboyim-Talleux *et al.*, 1999).

Germination. Garin *et al.* (1997) reported that conversion of sweet cherry somatic embryos is rare; however, 0.4 μM IBA in induction medium (MS supplemented with 2–22.5 μM 2,4-D and 4.5 μM kinetin) has enhanced normal development and germination of sour cherry somatic embryos (Tang *et al.*, 2000).

5.1.2. Organogenesis

Peach. Adventitious shoots have been induced from callus derived from immature zygotic embryos of peaches (Hammerschlag *et al.*, 1985). Immature zygotic embryos excised from 70-day-old peach fruits of 'Sunhigh' and 'Suncrest' were cultured on semi-solid MS medium supplemented with 2.4 μM 2,4-D and 0.45 μM BA and then transferred to medium containing 0.27 μM NAA and 2.25 μM BA. Shoots were regenerated from callus after transfer to medium with 2.25 μM BA and 1.35 μM NAA. Regenerated shoots were rooted in darkness on semi-solid medium containing 28.5 μM IBA. Shoot organogenesis can also occur from cultured immature cotyledons excised from 70-day-old fruits of 'Bailey', 'Boone County', 'Suncrest' and 'Hann' peaches on semi-solid MS medium containing 25 g/l sucrose, 2.5 μM IBA and 7.5 μM thidiazuron (TDZ) (Mante *et al.*, 1989). Roots are produced in 50–70% of the regenerated shoots under light conditions on half-strength MS semi-solid medium with 2.5 or 5 μM IBA.

Shoots have been regenerated from mature cotyledons excised from cold (4°C)-stored seeds of peach rootstocks 'Nemaguard', 'Floridaguard' and 'Nemared' on semi-solid MS medium containing 25 g/l sucrose, 1.25 or 2.5 μM IBA and 6.25 or 12.5 μM TDZ in darkness (Pooler and Scorza, 1995). The regenerated shoots were maintained on semi-solid medium with 0.1 μM IBA and 1.0 μM BA, and shoots were rooted on half-strength semi-solid MS medium with 5 μM IBA. Adventitious peach shoots have been regenerated from callus derived from young leaves (1–2 mm long) on medium containing 9 μM BA and 0.54 μM NAA (Gentile *et al.*, 2002).

Plum. Adventitious shoots have been regenerated from mature cotyledons of European plum (*P. domestica*) on semi-solid MS medium supplemented with 12.5 μM TDZ and 2.5 μM IBA (Mante *et al.*, 1989). About 25% of shoots root on half-strength MS medium with 2.5 or 5 μM IBA. Shoots have also been regenerated from hypocotyl sections of cold (4°C)-stored seeds of European plum on semi-solid MS basal medium containing 7.5 μM TDZ and 2.5 μM IBA (Mante *et al.*, 1991). The shoots were rooted on half-strength semi-solid MS medium with 10 g/l sucrose and 2.5 μM IBA. Novak and Miczynski (1996) regenerated shoots from mature phase plum leaves on MS medium with 7.5 μM TDZ and 1 μM 2,4-D. These leaf explants were excised from *in vitro*-grown adult shoot cultures on semi-solid MS medium with 1.12 μM BA and 0.4 μM IBA. Escalettes and Dosba (1993) observed that 10–40 μM silver nitrate increases shoot regeneration of *P. domestica*.

Cherry. Mature cotyledons excised from cold (4°C)-stored seeds of 'Montmorency' sour cherry (*P. cerasus*) produce adventitious shoots on semi-solid MS medium with 2.5 μM IBA and 7.5 or 10 μM TDZ (Mante *et al.*, 1989). Three to 5 mm long leaves from *in vitro*-grown shoot cultures of *P. avium* produced adventitious shoots on semi-solid MS medium with 0.54 μM NAA and 4.5 μM BA. The surfactant Tween 20 (10 mg/l) significantly improves shoot regeneration from leaf explants (Hammatt and Grant, 1998; Grant and

Hammatt, 2000). Hokanson and Pooler (2000) regenerated shoots from callus derived from mature cotyledons of *P. maackii*, *P. virginiana* and *P. serrula* on semi-solid MS medium with 2.5 μM IBA and 12.5 μM TDZ in darkness at 23°C. Development of shoots occurred after transfer of callus on to fresh medium under light conditions (16 h photoperiod). Tang *et al.* (2002) regenerated shoots from leaves of *in vitro* plants of sweet cherry 'Early Burlat', 'Hedelfinger', 'Napoleon' and 'Schneiders' and sour cherry 'Morellenfeuer' and 'Beutal Spacher Rexelle'. Proliferating cultures were established and maintained on semi-solid MS basal medium with 4.5 μM BA and 0.4 μM IBA under a 16 h photoperiod. Basal sections of the fully expanded leaves (5–8 mm long) produced shoots on WPM (Lloyd and McCown, 1981) with 9 μM BA and 2.7 or 5.4 μM NAA. The regenerated shoots were rooted in half-strength semi-solid MS medium containing 8 μM IBA or 10.8 μM NAA.

Apricots. Adventitious shoots developed from cotyledons of explanted immature zygotic embryos of 'Sundrop' apricot on semi-solid MS medium supplemented with 5 μM BA and 1 μM 2,4-D (Lane and Cossio, 1986). Laimer da Câmara Machada *et al.* (1992) induced adventitious shoots from cotyledons of immature zygotic embryos from 68–89-day-old fruits of 'Kecskemeter' apricot on semi-solid MS medium containing 20 g/l sucrose, 7.5 μM TDZ and 2.5 μM IBA. Shoot regeneration was induced from young expanded leaves excised from *in vitro*-grown cultures of two apricot cultivars on Quoirin *et al.* (1977) medium supplemented with 7.5 μM TDZ, 0.27 μM NAA and 30 μM silver nitrate in darkness (Escalettes and Dosba, 1993; Perez-Tornero *et al.*, 2000b).

Almond. Shoots have been regenerated from leaf explants of adult almond trees on semi-solid MS medium with 5.9 μM TDZ and 2.85 μM indoleacetic acid (IAA), whereas juvenile leaf cultures required 6.81 μM TDZ and 2.85 μM IAA for shoot induction (Miguel *et al.*, 1996). The uppermost expanded leaves from 4-week-old micropropagated shoots of 'Ne Plus Ultra' and 'Nonpareil' almond produced nodular callus and adventitious shoot buds

on semi-solid Almechdi and Parfitt (1986) medium with 9.8 μM IBA and 6.8 or 22.5 μM TDZ. Inclusion of 1% casein hydrolysate in the medium increased the amount of nodular callus as well as shoot regeneration frequencies of both almond cultivars (Ainsley *et al.*, 2000). For shoot regeneration, leaf cultures were maintained in darkness for 3 weeks and then exposed to light. Ainsley *et al.* (2001) also induced adventitious shoots from cotyledons of zygotic embryos excised from 100–115-day-old fruits of 'Ne Plus Ultra', 'Nonpareil', 'Carmel' and 'Parkinson' on semi-solid MS medium supplemented with 10 μM TDZ followed by culture on medium without growth regulators.

5.1.3. Haploid recovery

Callus has been established from cultured anthers of sour cherry and peach (Seirlis *et al.*, 1979; Hammerschlag, 1983); however, no haploid plants were regenerated. Twenty-four haploid plants were visually selected from 20,000 seedlings of open-pollinated and crosses of several peach genotypes (Toyama, 1974), and Scorza and Pooler (1999) produced F_1 hybrids of doubled haploids. Pollen of the 'Stanley' plum was subjected to γ -irradiation from a ^{60}Co source at 200 Gy/min, and 'Rainha Cláudia Verde' was pollinated with irradiated pollen. The presence of diploid endosperm seeds indicated that double fertilization did not occur, but embryos were not recovered (Peixe *et al.*, 2000).

5.1.4. Protoplast isolation and culture

Regeneration of plants from protoplasts has been reported for few *Prunus* spp. (see review by Ochatt and Patat-Ochatt, 1995), but this protocol has not been used by others. Plants were first regenerated from mesophyll protoplasts of the cherry rootstock 'Colt' (*P. avium* $\times$ *pseudocerasus*) and later from protoplasts derived from root cell suspensions of several cherry clones (Ochatt *et al.*, 1987). Calluses produced from *in vitro*-grown roots of 'Colt' cherry on semi-solid MS basal medium supplemented with 10.4 μM NAA and 2.25 μM BA were used to develop cell suspensions in

liquid medium of the same composition. Both leaves and cell suspensions were plasmolysed for 1 h in CPW 13M solution before protoplast isolation in enzyme solution containing (w/v) 1% Onozuka R-10, 0.2% Macerozyme R-10, 0.1% Driselase, 1% polyvinylpyrrolidone (MW 10,000), 2% Meicelase and 2% Rhozyme HP-150. The protoplasts were cultured in liquid MS medium supplemented with 9% mannitol and NAA and BA or zeatin. Initial protoplast division and colony formation occurred in MS medium with 4.5 or 9 μ M NAA plus 2.25 or 4.5 μ M BA or zeatin. Cell colonies developed in semi-solid MS medium containing 10.8 μ M NAA, 2.25 μ M BA and 2.25 μ M zeatin, and produced shoot buds after plating on to semi-solid MS medium with 0.54 μ M NAA, 3.3 μ M BA, 0.45 μ M zeatin and 50 mg/l casein hydrolysate. Reducing phenolic oxidation in *P. avium* mesophyll protoplast cultures by the addition of glycine resulted in plant regeneration (Ochatt, 1991). A similar protocol was used to regenerate plants from protoplasts of the ornamental species *P. spinosa* (Ochatt, 1992).

5.2. Genetic manipulation

5.2.1. Mutation induction and somaclonal variation

Natural and induced mutations have produced valuable peach cultivars (see review by Scorza and Sherman, 1996). The 'Compact Redhaven' peach is a bud mutation of 'Redhaven' (Van Well, 1974), in which the compact growth habit is controlled by a single dominant allele (Mehlenbacher and Scorza, 1986). Gamma-irradiation of 1-year-old peach cultivars at the Brookhaven National Laboratory produced late-ripening mutants of 'Fairhaven', 'Elberta' and a late-ripening clingstone mutant 'Brackett' (Scorza and Sherman, 1996). 'Galaxy' is an irradiation-induced mutant of 'Montmorency' cherry that produced more fruits on spurs (Okie and Weinberger, 1996). Thermal neutron irradiation created the early-ripening apricot mutant cultivar 'Early Blenheim' (Layne *et al.*, 1996). Two bud sports of 'Nonpareil' almond, the later blooming

'Tardy Nonpareil' and a red-kernelled bud sport, 'Kern Royal', were released for cultivation but did not become popular due to yield reductions (Kester and Gradziel, 1996). The almond cultivar 'Jeffries' is a mutant of 'Nonpareil', which has a dysfunctional S locus in both the pistil and the pollen (Ushijima *et al.*, 2001). Induced mutations have produced self-fertile clones and spur-type or compact tree types in cherry (Brown *et al.*, 1996).

The use of *in vitro* screening to select somaclonal variants of peach with resistance to bacterial diseases has been reviewed (Hammerschlag, 1990; Hammerschlag and Ognjanov, 1990). Somaclonal variants of peach plants resistant to bacterial leaf spot, caused by *X. campestris* pv. *pruni*, were selected from open-pollinated zygotic embryo callus of leaf spot disease-susceptible 'Sunhigh', as well as moderately resistant 'Redhaven'. Somaclonal variants were selected by exposing highly regenerative calluses to culture filtrates of *X. campestris* pv. *pruni* (Hammerschlag, 1990). Regenerants from two open-pollinated zygotic embryos of 'Sunhigh' and three embryos of 'Redhaven' were screened for resistance. The frequency of variation within different sets of multiple regenerants derived from a single embryo was significantly different, which suggests that the frequency of somaclonal variation is genotype-dependent. Hashmi *et al.* (1997) screened peach regenerants from 'Sunhigh' and 'Redhaven' embryos with 60 RAPD primers to identify somaclonal variants, thereby providing evidence for the existence of genetic differences among these variants. One regenerant each of 'Sunhigh' and 'Redhaven' showed polymorphism, indicating the possibility of using RAPD markers to identify somaclones of peach.

Of the 'Sunhigh' regenerants, 13% were significantly more resistant than control 'Sunhigh', while none of the 'Redhaven' regenerants were more resistant than the control. However, greenhouse and field evaluations of regenerated trees showed that two somaclones (No. 19-1 and No. 156-6) of 'Sunhigh' and one somaclone (No. 122-1) of 'Redhaven' showed high resistance to

X. campestris pv. *pruni*, suggesting that the variation is stable (Hammerschlag *et al.*, 1994). A detached bioassay of 5-year-old somaclone 122-1 showed a high level of resistance to *X. campestris* pv. *pruni* strain XP1 and *Pseudomonas syringae* pv. *syringae* (Hammerschlag, 2000).

5.2.2. Somatic hybridization

Somatic hybrids between the sexually incompatible rootstocks wild pear (*Pyrus communis* var. *pyraster* L.) and Colt cherry (*P. avium* × *pseudocerasus*) have been reported (Ochatt *et al.*, 1989). Protoplasts of 'Colt' cherry from suspension cultures and mesophyll protoplasts of wild pear were electroporated as separate populations and chemically fused (Ochatt *et al.*, 1988). This strategy resulted in a unique heterokaryon and was the basis for selection of hybrids. Only three shoot buds could be cloned *in vitro* from seven hybrid calluses, and shoots were established from two of those buds and later rooted. The hybrid plants were intermediate for most morphological markers compared to the parents. All somatic hybrid plants had 58 chromosomes, equivalent to the entire complement of somatic chromosome numbers of the parents.

5.2.3. Genetic transformation

Major objectives of *Prunus* genetic transformation include resistance to diseases and pests, alteration of growth habit, regulation of fruit ripening and tolerance of abiotic stress (Callahan *et al.*, 1991; Scorza and Hammerschlag, 1992). Since *Prunus* cultivars are not true to type from seed and must be clonally propagated for commercial production, plant regeneration from somatic cells of elite selections is essential to preserve genetic integrity; however, there are only a few reports of regeneration of transgenic plants from transformed somatic cells of *Prunus* (Archilletti *et al.*, 1995; da Câmara Machado *et al.*, 1995; Miguel and Oliveira, 1999). At present, there is no uniform protocol for transformation of *Prunus*, but the protocol developed by Mante *et al.* (1991) for recovery of transgenic plums from hypocotyl sections

of cold (4°C)-stored plum seeds has provided consistent results.

Transformation of *Prunus* species has been reviewed by da Câmara Machado and da Camara Machado (1995), Scorza *et al.* (1995a), Rugini and Gutierrez-Pesce (1999), Srinivasan and Scorza (1999) and Scorza (2001) (Table 18.3.1). Production of transgenic *Prunus* trees largely depends on efficient regeneration from transformed cells. Poor regeneration efficiency is a serious problem for *Prunus* spp. (Scorza, 2001) and is affected by the method of transformation, *Agrobacterium* strain, transformation environment and antibiotic concentration used for selection.

Peach. At this time only one report describes the recovery of transgenic peach plants (Smigocki and Hammerschlag, 1991). Cytokinin-over-expressing transgenic peach plants containing the *Agrobacterium tumefaciens* isopentenylphosphotransferase (*ipt*) gene were regenerated from embryogenic cultures derived from zygotic embryos of 'Redhaven'. Cultures were transformed with a shooty mutant strain of *A. tumefaciens*, *tms328::Tn5*, which carries an octopine type Ti plasmid with a functional cytokinin gene and a mutated auxin gene. These cytokinin-over-expressing transgenic peach trees are dwarf in stature, produce more branches and have delayed senescence of leaves (Hammerschlag *et al.*, 1997; Hammerschlag and Smigocki, 1998).

Cherry. In the cherry rootstock 'Colt', the introduction of tumour-inducing (T)-DNA from *Agrobacterium rhizogenes* produced trees with significant reductions in plant height, internode length, leaf area and petiole length (Gutiérrez-Pesce *et al.*, 1998). Similarly, the rice phytochrome A (*phyA*) gene conferred a dwarfing effect when over-expressed in the transgenic 'Colt' rootstock (Muleo and Iacona, 1998; Negri *et al.*, 1998). Under far-red light, its expression included reduced apical dominance and internode extension and increased branching of transgenic shoots. Trees are currently being field-tested. Since plant regeneration from transformed cells has been difficult in *Prunus*, Brasileiro *et al.* (1991) suggested that wild-type

Table 18.3.1. Transformation of *Prunus* species.

Genotype	Explant	Transformation method and genes	Results	Reference
<i>P. persica</i> cv. Redhaven	Immature zygotic embryos	<i>A. tumefaciens</i> tms::Tn5	Cytokinin over-producing transgenic peach plants	Smigocki and Hammerschlag (1991)
<i>P. domestica</i> breeding selection 70146, Stanley, Bluebyrd	Seed hypocotyl sections	<i>A. tumefaciens</i> EHA101/pCGN7001 and pCGN7314	Transgenic plum plants expressed GUS and NPTII proteins	Mante et al. (1991)
<i>P. avium</i> cherry clone INRA 235	Internodes of <i>in vitro</i> -grown plantlets	<i>A. rhizogenes</i> A4, <i>A. tumefaciens</i> C58, 84.5, 82.139, B6806, Bo542 (co-inoculation of oncogenic and disarmed strains) pB1121	Nopaline strains C58 and 82.139 induced shoot regeneration with normal phenotype	Brasileiro et al. (1991)
<i>P. armeniaca</i> cv. Kecskeneter	Cotyledons from immature zygotic embryos	<i>A. tumefaciens</i> LBA4404/pBinGUSint or pBinPPV _{int}	Transgenic apricot plants containing plum pox virus (PPV) coat protein gene	Laimer da Câmara Machado et al. (1992)
<i>P. domestica</i> cv. Stanley and advanced breeding selections B69158 and B70146	Seed hypocotyl sections	<i>A. tumefaciens</i> C58/Z707 and EHA101/pGA482GG/PPV-CP	Transgenic plum plants expressed PPV coat protein NPTII, and GUS	Scorza et al. (1994)
<i>P. domestica</i> cv. Stanley and advanced breeding selection B70146	Seed hypocotyl sections	<i>A. tumefaciens</i> C58/Z707/pGA482GG/CPPRV-4	Transgenic plum plants expressed papaya ringspot virus (PRV) coat protein	Scorza et al. (1995b)
<i>P. subhirtella autumnno rosa</i> cherry rootstock	Somatic embryogenic callus from petioles	<i>A. tumefaciens</i> LBA4404/pBinGUSint	Transgenic plants expressed GUS	da Câmara Machado et al. (1995)
<i>P. avium</i> × <i>P. pseudocerasus</i> rootstock Colt	Root	Inoculation of root with <i>A. rhizogenes</i> whole T-DNA pRi 1855	Somatic embryos and transgenic plants regenerated from transgenic roots	Gutiérrez-Pesce et al. (1998)
<i>P. dulcis</i> almond cvs Supernova and Boa Casta	Seedling leaves	<i>A. tumefaciens</i> LBA4404, and EHA105/pBinGUSint. PFAJ3003	Transgenic almond plants expressing GUS	Miguel and Oliveira (1999)

GUS, glucuronidase; NPTII, neomycin phosphotransferase II.

Agrobacterium could be combined with a disarmed strain where an optimal hormone balance for shoot regeneration would be supplied by the oncogenic T-DNA. Using this strategy, a separate regeneration procedure would not be needed. The transformation of roots of cherry rootstocks (F12/1) and plums (MRS/2) with a wild strain of *A. rhizogenes* or *A. tumefaciens* carrying *rolABC* genes produced transgenic shoots from roots (Rugini and Mariotti, 1992; Rugini and Gutierrez-Pesce, 1999). Transgenic sour cherry lines containing the anti-freeze protein (AFP) gene to reduce ice-crystal formation at freezing temperatures have been reported; however, phenotypic characteristics intrinsic to the AFP gene could not be detected (Dolgov, 1999). Da Câmara Machado *et al.* (1995) regenerated 110 transgenic lines of cherry rootstock (*P. subhirtella autumnosa*) using neomycin phosphotransferase II (*nptII*) as a selection marker. The use of hygromycin phosphotransferase (*hpt*) as a selection marker improved cherry transformation compared to *nptII* (Dolgov, 1999).

Almond. Miguel and Oliveira (1999) regenerated transgenic almond clones expressing both *nptII* and glucuronidase (*gus*) genes by transforming leaf explants of 'Boa Casta' seedlings. *Agrobacterium* strain EHA105 produced a higher percentage of GUS-positive plants than LBA4404 strain. Delaying kanamycin selection for 6 days also improved transformation efficiency of almond leaves (Archilletti *et al.*, 1995).

Apricot. Laimer da Câmara Machado *et al.* (1992) regenerated transgenic plants derived from cotyledons of 'Kecskemeter', which contained the PPV coat protein (PPV-CP) gene (Table 18.3.1). Transgenic apricots expressing virus coat protein genes showed tolerance of PPV disease (Rugini and Gutierrez-Pesce, 1999).

Plum. Transgenic clones of European plum seedlings containing the PPV-CP gene were produced from transformed hypocotyl sections (Scorza *et al.*, 1994). One transgenic clone, C5 (Fig. 18.3.2), is highly resistant to PPV (Ravelonandro *et al.*, 1997). Transgenic

plum plants expressing papaya ringspot virus coat protein (PRV-CP) delayed PPV symptoms but were not resistant to PPV, suggesting that a heterologous coat protein such as PRV-CP may not provide resistance to PPV (Scorza *et al.*, 1995b). The inheritance and expression of the PPV-CP gene as well as the GUS and *nptII* genes in C5 have been studied in the USA, France, Poland, Spain and Romania (Scorza *et al.*, 1998; Ravelonandro *et al.*, 2000). The PPV resistance in C5 has been stable and progenies produced through hybridization of transgenic plants with non-transgenic plums inherited and expressed the transgenes and PPV resistance (Ravelonandro *et al.*, 1998; Scorza *et al.*, 1998). The C5 plants displayed post-transcriptional gene silencing (PTGS) characterized by a high level of transgene transcription in the nucleus, low levels of transgene mRNA in the cytoplasm and methylation of the PPV-CP transgene



Fig. 18.3.2. High-quality plum fruits from plum pox virus-resistant transgenic clone C5 (Scorza *et al.*, 2001b).

(Scorza *et al.*, 2001b). This is the first report of PTGS in a temperate fruit tree. The C5 clone is resistant to all four major serotypes of PPV. Comparison of the nucleotide sequence of the capsid gene for these PPV serotypes showed that the two-thirds of the C-terminus sequence of the CP cistron could be responsible for the homology-dependent resistance (Ravelonandro *et al.*, 2001). The C5 clone could serve as a model to study virus resistance strategies in transgenic fruit trees (Ravelonandro *et al.*, 2000).

To improve rooting of difficult-to-root species, genetic transformation has been used to produce chimeric plants. This procedure has involved inoculation of the basal end of *in vitro*-grown shoots with a wild type of *A. rhizogenes* or *A. tumefaciens* carrying the *rol* genes of *A. rhizogenes*. This method has induced rooting in cherry and plum rootstocks (Rugini and Mariotti, 1992; Rugini and Gutierrez-Pesce, 1999), three plum cultivars (Bassi *et al.*, 1993; Monticelli *et al.*, 1997), *P. pennsylvanica* and *P. cerasus* (Haapala *et al.*, 1994).

5.3. Cryopreservation

Prunus cultivars can be cryopreserved with or without cryoprotectants and vitrification. *In vitro*-grown shoot tips of cherry (*P. jamasakura* Seib.) have been cryopreserved by one-step vitrification (Niino *et al.*, 1997). Shoot tips are excised from shoot cultures that have been cold-hardened for 45 days at 5°C. After 1 day on MS medium containing 0.7 M sucrose, the shoot tips are vitrified for 105 min at 25°C in plant vitrification solution 2 (PVS2) formula (Sakai *et al.*, 1991), consisting of glycerol (30%), ethylene glycol (15%) and dimethyl sulphoxide (DMSO) (15%). The shoot tips are immersed directly into liquid nitrogen. About 80% of the cryopreserved shoot tips of seven cherry cultivars have survived for up to 10 months following partial desiccation and then cooling to about -160°C by a two-step procedure. A procedure without cryoprotectants has been used by Towill and Forsline (1999) for dormant vegetative buds of sour cherry. The shoots are desiccated to 25% moisture content,

cooled at 1°C/h to -30°C before immersion in -160°C. Approximately 40% of five sour cherry scions survived as evaluated by chip budding to rootstocks.

Shoot-tip vitrification and encapsulation-based procedures have been developed for plum cryopreservation (De Carlo *et al.*, 2000). The shoot tips are precultured at 4°C for 2 days on 0.09 M sucrose medium, loaded for 30 min with a cryoprotectant solution (2 M glycerol and 0.4 M sucrose) and vitrified at 0°C for 90 min in PVS2 before plunging into liquid nitrogen.

Shoot apices of *in vitro*-grown plantlets of almond rootstock M51 and 'Faargnes' have been cryopreserved by vitrification and by encapsulation-dehydration (Shatnawi *et al.*, 1999). The highest survival rate (50–62%) was achieved by encapsulation-dehydration. Optimum conditions include pregrowth of encapsulated apices for 1–3 days in liquid medium with 0.75 M sucrose and desiccation of beads to 19–20.3% moisture content followed by direct immersion in liquid nitrogen.

6. Conclusions

The genetic improvement of *Prunus* species through conventional breeding is a long-term and expensive process, but the success of this approach is apparent in the large number of cultivars that are being grown commercially that have been released from breeding programmes, especially in the case of peach. Biotechnological approaches offer the potential of shortening the time required for cultivar development and making genetic improvements that are impossible through conventional means. Advances in genetic marker development, regeneration, gene identification, gene transfer, micropropagation and cryopreservation are promising. At the same time, there are significant blocks to progress in all of these areas. These are particularly evident in the area of regeneration and transformation, which are keys to the development of improved existing cultivars with traits that cannot be readily found in the existing germplasm. The development

of PPV-resistant plums presents an example of the potential benefit of this technology. Strong interdisciplinary programmes that incorporate classical and new genetic improvement technologies will be critical for the future development of improved *Prunus* cultivars that can tolerate adverse biotic and abiotic conditions and provide income for growers and products appreciated by consumers.

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18.4 *Pyrus* spp. Pear and *Cydonia* spp. Quince

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1. Introduction

1.1. Botany and history

The genus *Pyrus* belongs to the subfamily *Maloidae* in the *Rosaceae*, and contains 22 widely recognized primary species, all indigenous to Europe, temperate Asia and the mountainous areas of North Africa (Westwood, 1982). The genus is believed to have arisen during the Tertiary period in the mountainous regions of western China. Dispersal and speciation are believed to have followed the mountain chains both east and west (Rubzov, 1944; Zeven and Zhukovsky, 1975). There are at least six naturally occurring interspecific hybrids. Economic usage of *Pyrus* species has been reviewed by Bell *et al.* (1996a). Cultivars of *P. communis* L. are the main edible pears in Europe, North America, South America, Africa and Australia. The snow pear, *P. nivalis* Jacq., is also grown to a limited extent in Europe for making perry. In Asia, *P. pyrifolia* (Burm.) Nakai is the main cultivated species in southern and central China, Japan, Taiwan and countries of South-east Asia. Some cultivars of this species are also grown on a more limited basis in Europe, North America and elsewhere. In northern China and Japan, *P. ussuriensis* Max. and *P. × bretschneideri* Rehd. are grown (Kikuchi, 1946; Shen, 1980). Selections of *P. pashia* P. Don. are cul-

tivated in southern China (Pieniazek, 1966) and northern India (Mukherjee *et al.*, 1969). Species used as ornamentals include the small-fruited *P. calleryana* Decne., *P. fauriei* Schneid., *P. betulaeifolia* Bunge, *P. salicifolia* Pall. and *P. kawakamii* Hayata. Seedlings and clonal selections of *P. betulaeifolia*, *P. calleryana*, *P. pyrifolia*, *P. ussuriensis* and *P. communis* are used as rootstocks in Europe, North America and eastern Asia, and *P. pyrastrer* Burgsd., *P. amygdaliformis* Vill. and *P. elaeagrifolia* Pall. are occasionally used in Asia Minor and central Asia (Lombard and Westwood, 1987).

Pears rank second to apples in the amount of worldwide production of deciduous tree fruit species. World production was approximately 17.2 million t on 1.6 million ha (FAOSTAT, 2004). China produces more pears than any other country, with 9.4 million t on 953,800 ha although these are almost exclusively of the Asian species. The major producers of European pear (*P. communis*) are the USA (837,420 t), Italy (821,675 t), Spain (681,000 t) and Argentina (560,000 t). The value of world pear imports and exports in 1998 was approximately US\$1 billion (Segré, 2002). Increased production area and total crop size have been forecast for the next 4 years (Segré, 2002). Most of the crop is utilized as fresh fruit, but significant amounts are processed and marketed as canned pears, purée, juice or other products.

1.2. Breeding and genetics

The genera of the subfamily *Maloideae* have a basic chromosome number of 17, and may have arisen as an amphidiploid of two primitive forms of *Rosaceae*, one having a basic chromosome number of 8 and the other 9 (Sax, 1931; Zielinski and Thompson, 1967). These were possibly primitive members of the *Prunoideae* and *Spiraeoideae*, respectively. All species of *Pyrus* that have been examined are diploid ($2n = 34$; $x = 17$). No significant interspecific cross-incompatibility is known to exist (Bell and Hough, 1986).

1.2.1. Rootstocks

Trees of pear cultivars are usually asexually reproduced as clones by budding or grafting on to a genetically distinct rootstock, in contrast to being self-rooted. This compound tree system has the advantage that specific adaptations to soil conditions and pests, as well as root traits which can affect the performance of the scion, do not have to be part of the genetic potential of the scion. Pears are, to varying degrees, graft-compatible with other rosaceous genera, so in addition to seedlings of *P. communis* and other European species, clones of quince (*Cydonia oblonga* L.) have been extensively used as rootstocks for *P. communis* scion cultivars, particularly in Europe. In Asia, seedlings of *P. pyrifolia*, *P. betulifolia*, *P. calleryana*, *P. pashia*, *P. xerophila* and *P. kawakamii* have been used and, in Asia Minor and the Mediterranean regions, seedlings of *P. elaeagnifolia*, *P. syriaca*, *P. amygdaliformis* and *P. longipes* have been used.

Major breeding objectives. The usage and characteristics of existing rootstocks, as well as desirable characteristics and germplasm with potential as rootstocks have been reviewed by Lombard and Westwood (1987), Bell (1991), Bell *et al.* (1996a) and Bellini (1995). Breeding objectives have been reviewed by Lombard and Westwood (1987) and Webster (1998). The primary objectives of all rootstock breeding programmes have been the induction of precocious, high and annually consistent yields of large fruit. The ability to dwarf the trees has been of almost

equal importance, since small trees are easier to manage and harvest and can result in high yields per unit land area. Many of the major European pear (*P. communis*) scion cultivars are graft-incompatible on quince, resulting in graft union necrosis, weak growth and breakage. Therefore, graft-compatibility is an objective of quince rootstock breeding in Europe. Ability to root by stooling, hardwood cuttings or semi-hardwood cuttings is an important objective, since modern rootstocks are clonally propagated.

Adaptation to general temperature patterns of each production region, principally warm winter/hot summer climates, in which chilling requirement for overcoming dormancy is important, or to cold winter climates, in which acclimatization and cold hardiness are important, are specific objectives. Improved cold hardiness has been a breeding objective for *Pyrus* and *Cydonia* rootstocks in Europe. Adaptation to high soil pH, which can result in iron chlorosis, particularly in quince, is a major objective of breeding programmes in France, Italy and countries of southern Europe. Adaptation to soil moisture and texture are secondary objectives.

In Europe and North America, the primary disease problems of *P. communis* rootstocks are the bacterial disease fire blight, caused by *Erwinia amylovora* (Burr.) Winsl. *et al.*, and the pear decline phytoplasma. Crown gall (*Agrobacterium tumefaciens* (E.F. Sm. & Towns) Conn.) and collar rot (*Phytophthora cactorum* (Leb. & Cohn) Schroet.) are secondary objectives. The woolly pear aphid (*Eriosoma pyricola* Baker & Davidson) and the root knot nematode (*Meloidogyne* spp.) are the major pest problems, but they are not the explicit objectives of any breeding programme.

Black end is a physiological fruit disorder affecting both Asian and European pears when grown on *P. ussuriensis* and *P. pyrifolia* rootstocks. Lack of black end is an objective of Japanese and Chinese breeding programmes.

Breeding accomplishments. Progress in rootstock breeding for pears has been reviewed by Lombard and Westwood (1987),

Bellini (1995) and Webster (1998). Many new rootstocks are the result of open pollination, but deliberate evaluation and selection of parents and hybridization has become more common.

Several new promising *P. communis* rootstocks have been developed. In North America, clonal selections of a cross between two fire blight-resistant cultivars, 'Old Home' and 'Farmingdale' ('OH × F'), which provide improved levels of fire blight resistance and yield efficiency, combined with slight to moderate degrees of tree size control, have been introduced (Brooks, 1984; Lombard and Westwood, 1987). Currently available rootstock clones are 'OH × F 40', 'OH × F 51', 'OH × F 69', 'OH × F 87', 'OH × F 97', 'OH × F 232', 'OH × F 333' and 'OH × F 513'. From South Africa, BP1 induces a semi-dwarf size tree, but is difficult to propagate. The Brossier series, derived from perry pear (*P. nivalis*), yielded two clones, RV.139 and G.54-11, which induced dwarfing and good yield efficiency (Brossier, 1977; Michelesi, 1990), but which have proved to be very difficult to propagate, even *in vitro*. The Rétuzière series, derived from open pollination of 'Beurre Hardy', 'Old Home' and 'Kirchensaller', has produced 'Pyriam' (OH11) (Michelesi, 1990; Simard and Michelesi, 2002). Another recent cultivar, 'Pyrodwarf' (Rhenus 1), of 'Old Home' × 'Bonne Louise d'Avranche' parentage, appears to provide improved fire blight resistance, precocity, yield efficiency, size control and ease of propagation (Jacob, 1998). In Italy, there has been an emphasis on breeding *Pyrus* rootstocks to replace quince rootstocks, which are susceptible to low pH-induced iron chlorosis. Derived from the old Italian cultivar 'Volpina', two selections from this 'Fox' series are being more widely tested (Bassi *et al.*, 1996). The programme at Horticultural Research International (HRI)-East Malling has produced the 708 series from a cross of 'Old Home' and BP1. Three clones which produce trees smaller to slightly larger than Quince A are currently being more widely evaluated (Webster, 1998).

Quince selections with improved cold hardiness have been produced in Poland

(Webster, 1998; S. Zagaya, personal communication). Two clones selected in Italy, Ct.212 and Ct.214, are reported to be more tolerant of lime-induced chlorosis (Loreti, 1994). Selection at HRI-East Malling for very dwarfing rootstocks adapted to very high-density planting has produced C132, and QR 193-16 induces large fruit size.

1.2.2. Scion cultivars

General breeding objectives for scion cultivars throughout the world are remarkably similar, especially among European and Asian programmes. Information on objectives and recent introductions can be found in reviews by Bellini (1995), Bell *et al.* (1996a), Bellini *et al.* (2000) and Sansavini *et al.* (2000).

Major breeding objectives. Breeding objectives can be divided into the general categories of fruit quality and appearance, productivity, environmental adaptation and disease and insect resistance. The most important objective is eating quality, including flavour and texture. Flavour is increasingly being evaluated in terms of component traits (sugars and acids). Fresh fruit quality is of primary concern, but a few programmes also consider the quality of canned or puréed fruit. Some programmes target specific marketing periods, and time of harvest, storage, shelf-life and uniform ripening after storage are important selection criteria. Susceptibility to postharvest physiological disorders such as core breakdown and bitter pit, associated with low calcium ion content of the flesh, superficial scald, associated with high levels of α -farnesene, and other sometimes more cultivar-specific disorders, e.g. stony pear of *P. pyrifolia*, are also of major importance. Development of cultivars with novel textural and flavour characteristics are goals of programmes in the USA, New Zealand and Italy, which have employed interspecific hybridization of European and Asian germplasm.

Precocious cropping can be selected for in seedlings because of a high correlation with juvenile period. Annual high fruit yields are equally important for commercial viability. Since pears are highly self-incompatible,

selection for self-compatibility has recently become a focus of several programmes. Growth habit, principally through selection of natural or induced dwarf mutants, is seen as an alternative to dwarfing rootstocks.

Like rootstocks, adaptation to temperature extremes is important. Most production of pears is in temperate regions, and cold hardiness has been a major goal of programmes in Canada and northern Europe. Adaptation to more southern regions has usually involved germplasm with low chilling requirements, e.g. Asian pear species, *P. pyrifolia*, and interspecific hybridization.

In North America and much of Europe, resistance to fire blight is a major objective. Pear scab (*Venturia pirina* Aderh.) is an objective, especially in northern Europe. In China, Japan and Korea, resistance to the Asian pear scab (*Venturia nashicola* Tan. & Yam.), black spot (*Alternaria alternata* [Fr.] Keissler) and pear rust (*Gymnosporangium haraeaeum* Syd) are important. Resistance to the pear psylla (*Cacopsylla pyricola* Foerster, *C. pyri* L. and *C. pyrisuga* Foerster), which, in addition to being a direct pest, is the vector of the pear decline phytoplasma, is an objective of breeding programmes in North America, France, Romania, Poland and Italy.

Breeding accomplishments. Many new scion cultivars have been developed and released within the last 20 years (Bellini, 1995; Bellini *et al.*, 2000), and are too numerous to mention individually. Major improvements have been made in the quality, storage and shelf-life of early-season pears, as well as in late-season pears (Bellini *et al.*, 2000). Selection for dual-purpose cultivars with 'Bartlett'-type flavour and aroma has resulted in the release of 'Harvest Queen' in Canada (Quamme and Spearman, 1983). Several cultivars with improved storage life and postharvest quality have also been released from European programmes. The New Zealand programme has named interspecific hybrids with crisp flesh (White and Brewer, 2002).

Interspecific *P. communis* × *P. pyrifolia* hybrids adapted to low chilling hours have been released by the University of Florida.

With the exception of 'Garden Pearl' (Bellini *et al.*, 2000), genetically dwarfed scion cultivars have not been commercially introduced, although programmes in Italy (Rivalta *et al.*, 2002) and France (Le Lezec, personal communication) have been breeding for reduced stature, using the short-internode dwarf 'Le Nain Vert' as a source of dwarfing.

Recent fire blight-resistant pear cultivar releases include 'Potomac' (Bell *et al.*, 1996b), 'Blake's Pride' (Bell *et al.*, 2002), 'Harvest Queen', 'Harrow Delight' (Quamme and Spearman, 1983), 'Harrow Sweet' (Hunter *et al.*, 1992), 'Harrow Gold' (Hunter *et al.*, 2002a) and 'Harrow Crisp' (Hunter *et al.*, 2002b), and other breeding programmes in Italy, France and Romania have selections with improved resistance. Selection for a lack of secondary or late blossoms is a selection strategy employed with some success by both the Institut National de la Recherche Agronomique (INRA) and HRI programmes to reduce susceptibility. Resistance to pear scab in European pears is now found in new cultivars from Germany (Fischer and Mildenerberger, 1999) and Romania (Bellini *et al.*, 2000). Cultivars of *P. pyrifolia* such as 'Hosui' are resistant to black spot (Kanato *et al.*, 1982), and an irradiation-induced mutant of the susceptible cultivar 'Nijisseiki' has been developed (Sanada *et al.*, 1988) and released as a cultivar (Kajiura, 1992). Improved resistance or tolerance to pear psylla has been reported in the Romanian cultivars 'Haydeea', 'Euras', 'Ina Estival' and 'Getica' (Braniste, 2002). Additional sources of resistance from both Asian (*P. ussuriensis*) and East European (*P. communis*) cultivars and wild germplasm have been identified and are being used in breeding (Bell and van der Zwet, 1998).

2. Molecular Genetics

2.1. Gene cloning

The first gene cloned from the pear genome was a complementary DNA (cDNA) encoding a polygalacturonase inhibitor protein (PGIP) (Stotz *et al.*, 1993). This family of glycoproteins, which specifically inhibit fungal

polygalacturonase activity, has been proposed as a component of the general defence mechanism of plants against pathogens. Another gene which could have a function in plant defence has also been cloned, and encodes a thaumatin/PR5-like protein (Sassa and Hirano, 1998). Several studies have focused on the isolation of genes expressed during fruit development or ripening. 1-Aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase genes have been cloned from *P. communis* (Lelièvre *et al.*, 1997). The expression of other enzymes has been studied during pear fruit ripening: alcohol dehydrogenase (Chervin *et al.*, 1999) and polyphenol oxidase (Haruta *et al.*, 1999). Several genes from *P. pyrifolia* that are expressed during fruit ripening have been cloned: ACC synthase (Itai *et al.*, 1999a) and β -D-xylosidase, which could be a senescence-related protein (Itai *et al.*, 1999b). Genes encoding membrane-bound proteins which could be involved in fruit development have also been characterized from pear tissue: arabinogalactan proteins (Mau *et al.*, 1995) and *H*(+)-pyrophosphatase (Suzuki *et al.*, 1999). A cDNA fragment encoding a β -galactosidase, a key component in fruit softening, has been isolated from *P. pyrifolia* 'Hosui' (Tateishi *et al.*, 2001).

Another important field of molecular studies in Asian pears has been the self-incompatibility (*S*) locus. cDNAs from several *S* ribonucleases (*S*-RNases) have been cloned (Sassa *et al.*, 1996; Sassa and Hirano, 1997; Ushijima *et al.*, 1998) as well as a mutated allele of the *S4*-RNase conferring self-compatibility in the cultivar 'Osa-Nijiisseiki' (Norioka *et al.*, 1996). The gene encoding a uridine diphosphate (UDP)-glucose pyrophosphorylase of Asian pear, probably involved in pollen tube wall synthesis, has also been cloned (Kiyozumi *et al.*, 1999). A sequence encoding an RNA polymerase has also been identified from Japanese pear (Osaki *et al.*, 1998). In addition, a number of partial or complete cDNA sequences from pear and quince have been submitted to nucleotide databases (Table 18.4.1). Most of them have been isolated from fruits of Asian pear cultivars.

2.2. Molecular markers

Various types of genetic markers have been developed for pear. Peroxidases were the first isozyme markers and were applied to *P. calleryana* (Santamour and Demuth, 1980). Several other enzyme systems have been used for cultivar identification in different species of *Pyrus* and *Cydonia*. The more recent publications on pear isozymes described the cultivar identification of 50 Asian pears (Chung and Ko, 1995) and the use of isozymes for pollination studies (Sharifani and Jackson, 1997). Inheritance of 22 isozyme loci has now been established and their linkage relationships have revealed a high degree of synteny between apple and pear (Chevreau *et al.*, 1997b). RFLP markers of mitochondrial DNA were applied to study phylogenetic relationships among *Pyrus*, *Malus* and *Cydonia* (Iketani *et al.*, 1993). Restriction fragment length polymorphism (RFLP) analyses of chloroplast DNA (Iketani *et al.*, 1998) and of genomic DNA (Kawata *et al.*, 1995) have also been applied to the study of genetic diversity and taxonomic relationships. Random amplified polymorphic DNA (RAPD) markers were also developed for cultivar identification by several groups (Botta *et al.*, 1998; Gerlach and Stosser, 1998; Oliveira *et al.*, 1999; Banno *et al.*, 2000) and sequence-characterized amplified region (SCAR) markers were derived recently from RAPD markers (Kim *et al.*, 2000a). A comparison of RAPD, amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSR) and selective amplification of microsatellite polymorphic loci (SAMPL) markers was recently reported (Monte-Corvo *et al.*, 2000a,b; Yamamoto *et al.*, 2001). Most of these studies on molecular markers have been concerned with cultivar identification or phylogenetic relationships (Table 18.4.2). Only three recent reports, all involving Asian pears, deal with markers specifically linked to a gene of interest. Banno *et al.* (1999) used the bulk segregant analysis method to identify an RAPD marker at 3 cM of the *A* gene, controlling susceptibility to black spot disease (*A. alternata*) in Asian pears. Ishimizu *et al.* (1999) identified the *S* locus genotypes of Asian pears (seven

Table 18.4.1. Pear gene sequences in database NCBI-ENTREZ (unpublished).

Gene	Product	Origin: genotype/organ	Type of sequence	Locus number	Date	Author
bgn-1	Endo-1,3-β-glucanase	<i>P. pyrifolia</i> (cv. Chojuro), pollen	Complete	AB052291	Apr. 01	Zhou <i>et al.</i>
Py-PIP1-1	Plasma membrane intrinsic proteins (aquaporins)	<i>P. communis</i>	Complete	AB058679	Mar. 01	Kobae <i>et al.</i>
Py-PIP2-1			Complete	AB058678		Goto <i>et al.</i>
Py-PIP2-2			Complete	AB058680		Shiratake <i>et al.</i>
JPBGAL	β-galactosidase	<i>P. pyrifolia</i> (cv. Hosui), fruit	Complete	AB046543	Jan. 01	Tateishi <i>et al.</i>
PyPSUS1	Sucrose synthase	<i>P. pyrifolia</i> (cv. Hosui), fruit	Complete	AB045710	Jan. 01	Tanase <i>et al.</i>
Py-gTIP	γ-tonoplast intrinsic protein	<i>P. communis</i>	Complete	AB048248	Sep. 00	Shiratake <i>et al.</i>
PPFRU36	F1-ATPase	<i>P. pyrifolia</i> (cv. Kikusui), fruit	Partial	AB021794	Jun. 00	Itai <i>et al.</i>
PPFRU32	Asparagine synthetase		Partial	AB021793		
PPFRU21	Absciscic stress ripening protein		Partial	AB021792		
PPFRU19	Polygalacturonase inhibitor protein		Partial	AB021791		
PPFRU16	Metallothionein-like protein		Complete	AB021790		
PPFRU14	Calmodulin		Partial	AB021789		
PPFRU13	Glycine-rich protein		Partial	AB021788		
PPFRU11	Aspartic endopeptidase		Partial	AB021787		
PPFRU9	Endo-chitinase class III		Partial	AB021786		
PPFRU8	Metallothionein-like protein		Complete	AB021785		
PPFRU7	Cysteine protease		Partial	AB021784		
S3-RNase	Ribonuclease	<i>P. pyrifolia</i> (cv. Chojuro)	Complete	D49529	Apr. 00	Norioka <i>et al.</i>
PYPRBCO	Rubisco small subunit	<i>P. pyrifolia</i> , leaf	Complete	D00572	Feb. 00	Kano-Murakami
PYPLHABBP	Light harvesting a/b binding protein		Complete	D00571		
–	Internal transcribed spacer 1 and 2 of the 5.8S ribosomal RNA	<i>Cydonia oblonga</i>	Complete	AF186531	Dec. 99	Robinson <i>et al.</i>
–	18S ribosomal RNA	<i>P. communis</i> (cv. Winter)	Partial	AF195622	Nov. 99	Kim <i>et al.</i>
JPROMT	O-methyltransferase	<i>P. pyrifolia</i> (cv. Nikisseiki), fruit	Complete	AB014456	Nov. 99	Itai <i>et al.</i>
PyRC4	Profilin (allergen)	<i>P. communis</i> (cv. Williams)	Complete	AF129424	Aug. 99	Scheurer <i>et al.</i>
PyRC5	Isoflavone reductase related (allergen)		Complete	AF071477	Aug. 99	Karamloo <i>et al.</i>
PyRC1	Major allergen		Complete	AF057030	Apr. 98	Karamaloo <i>et al.</i>
–	Cysteine protease inhibitor	<i>P. communis</i> (cv. Passe Crassane)	Complete	U82220	Sep. 97	Gaillard <i>et al.</i>

Rubisco, ribulose 1,5-bisphosphate carboxylase.

Table 18.4.2. Molecular marker studies in pear since 1992.

Type of marker	Plant material	Aim of the study – main results	References
Isozymes	PER, GPI, GOT PER, MDH, EST	Identification and classification Identification, Red Bartlett distinguished from Bartlett	Chung and Ko, 1995 Hudina <i>et al.</i> , 1996
	GPI, IDH, PGM, ADH, MDH 11 enzymes β-GLUC, EST, PER	Segregation patterns, control of pollen flow Inheritance of 22 loci Modification of patterns linked to the nature of the rootstock	Sharifani and Jackson, 1997 Chevreau <i>et al.</i> , 1997b Fachinello <i>et al.</i> , 1999
	RFLP	Phenetic clustering Haplotype determination Identification and classification Marker of the climacteric trait	Iketani <i>et al.</i> , 1993 Iketani <i>et al.</i> , 1998 Kawata <i>et al.</i> , 1995 Itai <i>et al.</i> , 1999a
RAPD	– 20 primers 22 primers, 327 polymorphic bands Isozymes + RAPD 250 primers for BSA	Identification Identification Identification and clustering according to the geographic origin Identification of parenthood of Kuratsuki Identification of a marker linked to black spot disease resistance	Gerlach and Stosser, 1998 Botta <i>et al.</i> , 1998 Oliveira <i>et al.</i> , 1999 Banno <i>et al.</i> , 2000 Banno <i>et al.</i> , 1999
	6 RAPD markers 18 SrDNA sequences S-allele digestion of a specific PCR fragment	Identification of 6 SCAR markers Similarity analysis and clustering Identification of S-genotype of Asian pears	Kim <i>et al.</i> , 2000a Kim <i>et al.</i> , 2000b Ishimizu <i>et al.</i> , 1999
	AFLP ISSR	Comparison of markers for identification and clustering Identification and synteny with apple cultivars	Monte-Corvo <i>et al.</i> , 2000a,b, 2001 Yamamoto <i>et al.</i> , 2001

SSR, simple sequence repeat; PER, peroxidase; GPI, glucose phosphate isomerase; GOT, glutamate oxaloacetate isomerase; MDH, malate dehydrogenase; EST, esterase; IDH, isocitrate dehydrogenase; PGM, phosphoglucumutase; ADH, alcohol dehydrogenase; β-GLUC, β-glucosidase.

alleles) by polymerase chain reaction (PCR) and specific restriction. Itai *et al.* (1999a) established a correlation between the expression of two different ACC synthase genes (identified by their RFLP profiles) and ethylene formation in 35 Asian pear cultivars, some climacteric and some not. A linkage map of Asian pear has been published (Iketani *et al.*, 2001). Based on the segregation of more than 100 RAPD markers, 18 and 22 linkage groups were constructed for the two parents ('Kinchaku' and 'Kosui', respectively). The resistance allele of pear scab (*Vn*) and the susceptible allele of black spot (*A*) were mapped in different linkage groups. Recently, genetic linkage maps of both European and Asian pears were constructed based on an interspecific cross. Comparison with maps of other species belonging to the *Rosaceae* was made (Yamamoto *et al.*, 2002).

3. Micropropagation

Pear micropropagation was initiated with the first report of micropropagation of a pear rootstock, 'OH × F51' (Cheng, 1979) and a pear cultivar, 'Bartlett' (Lane, 1979). Since these first reports, significant progress has been made in pear micropropagation (Singha, 1986; Chevreau *et al.*, 1992) as well as in that of quince (Duron *et al.*, 1989). The latest achievements concern the adaptation of micropropagation techniques to various species of the genus *Pyrus*: *P. calleryana* (Berardi *et al.*, 1993), *P. calleryana*, *P. betulacifolia* (Yeo and Reed, 1995) and *P. syriaca* (Shibli *et al.*, 1997).

3.1. Establishment of cultures

Pear cultures are initiated from shoot tips or single node explants from grafted plants grown in the greenhouse. Shoot tips or buds taken from field-grown trees are usually more difficult to disinfect. Surface sterilization is achieved by immersion in a sodium hypochlorite solution (0.5–2.5%) containing a wetting agent (Tween-20 at 0.01%) for 5–30 min. Explants are then rinsed three times in sterile water. The size of the explants can

vary from the meristematic dome surrounded by two- to four-leaf initials to a 2 cm nodal or apical explant. Better results are generally obtained with explants taken from actively growing plants (Chevreau *et al.*, 1992). A single hypochlorite treatment is usually insufficient to ensure contaminant-free cultures. A two-step procedure with increasing doses of hypochlorite at 24 h intervals can improve the decontamination.

3.2. Axillary bud proliferation and shoot development

Proliferation rates of from two to ten shoots per explant have been reported for both European and Asian pears. The conditions for proliferation have been summarized by Chevreau *et al.* (1992), and include Murashige and Skoog (1962) (MS) revised medium, with benzyladenine (BA) (4.4–8.8 μ M) and either indolebutyric acid (IBA) (0.5 μ M) or naphthaleneacetic acid (NAA) (0.05–0.1 μ M). Cultures are grown at 22–28°C, under a 16 h photoperiod. Viseur (1987) and Rodriguez *et al.* (1991) recommended the use of a double-phase medium. This system was further refined by Damiano *et al.* (1999), who successfully applied a technique of temporary immersion of the explants in liquid medium to a wild pear (*P. communis* var. *pyraster*). More recently, Bommineni *et al.* (2001) proposed a micropropagation method based on multiple shoot regeneration from thin shoot slices, resulting from the reorganization of axillary meristems.

3.3. Rooting and acclimatization

Rooting and acclimatization of pear are difficult (Chevreau *et al.*, 1992). A dilute mineral medium with 0.1–10 μ M IBA or NAA has been used. In general, an initial period of 7–10 days of dark incubation improves rooting. A detailed study of auxin and polyamine metabolism was reported by Baraldi *et al.* (1995) to explain the differential behaviour of two easy- and difficult-to-root pear cultivars. The critical role of endogenous indole-3-acetic acid (IAA) levels was

demonstrated. Acclimatization can be accomplished in various substrates by progressively decreasing the relative humidity. Resumption of plant growth, which is often difficult, can be improved by foliar sprays of gibberellic acid (GA_3) (100–200 ppm). The use of a commercial inoculum of endomycorrhizae at the beginning of the weaning stage can also have a beneficial effect on the survival of pears (Bourrain *et al.*, 1999).

4. Micrografting

Micrografting of pear has been suggested to produce virus-free plants (Faggioli *et al.*, 1997). Seedlings of *P. communis* grown *in vitro* from disinfected seeds are used as rootstocks for apical meristems from potted mother plants grown in the greenhouse or from *in vitro*-grown plants. Rooted plantlets are ready for acclimatization 50–60 days after grafting. This technique is useful for eliminating pear vein yellow virus (PVYV) from infected pear cultivars.

5. Somatic Cell Genetics

5.1. Regeneration

5.1.1. Somatic embryogenesis

Janick (1982) reported the recovery of somatic embryos from immature seeds; however, there have been no other reports of somatic embryogenesis. Somatic embryogenesis from quince leaves has been studied by several groups (Antonelli, 1995; D'Onofrio *et al.*, 1998; Fisichella *et al.*, 2000). Factors such as hormonal combinations, light quality and macroelement composition were optimized. Induction of somatic embryogenesis was obtained from BA29 *in vitro* leaves through a two-step procedure: liquid medium with 2,4-dichlorophenoxy-acetic acid (2,4-D) (11 μ M) followed by culture on semi-solid medium supplemented with kinetin (4.6 μ M) and NAA (0.5 μ M). MS medium and red light illumination provided the most favourable conditions; however, somatic embryos were morphologically abnormal and failed to convert into plantlets.

5.1.2. Organogenesis

Efficient techniques for regenerating whole plants from leaves of *in vitro* elite cultivars through adventitious bud regeneration are available for a number of pear genotypes, including *P. communis* cultivars (Chevreau *et al.*, 1997a), *P. pyrifolia* cultivars (Lane *et al.*, 1998), *P. syriaca* Boiss. genotypes (Shibli *et al.*, 2000), *P. pyrastrer* rootstocks (Caboni *et al.*, 1999) and quince rootstocks (Dolcet-Sanjuan *et al.*, 1991; Baker and Bhatia, 1993). In all cases, leaves from *in vitro*-grown plants have been used as explants and have been exposed to a sequence of dark (2–4 weeks) and light exposure. The optimum culture conditions for adventitious regeneration are summarized in Table 18.4.3. These techniques involve a very limited callus phase at the site of wounding, and bud regeneration occurs in 3–6 weeks.

5.1.3. Haploid recovery

Pear is an allogamous species with a gametophytic self-incompatibility system. Selfing is very difficult to achieve and leads to a strong inbreeding effect. Much knowledge has been gained recently about the biochemical and genetic basis of incompatibility, particularly in Asian pears (Norioka *et al.*, 1996), and genetic engineering of the S locus has been proposed as a way to develop self-fertile cultivars (Van Nerum *et al.*, 2002).

Haploid embryos of Asian pears have been obtained from uninucleate anthers ('Jin Feng') and rooted plants in the field have been reported (Xue *et al.*, 1996). Haploidization studies involving European pear have been developed at INRA, Angers, with the aim of producing homozygous genotypes. Haploid pears have been produced by two different methods: (i) spontaneous haploid production among seedlings in the greenhouse and selection on the basis of specific phenotypical traits, followed by *in vitro* culture of the young haploid plantlets; and (ii) pollination with irradiated pollen followed by *in vitro* culture of the parthenogenetic immature embryos (Bouvier *et al.*, 1993). In both cases, *in vitro* rescue of the haploid plants was necessary but not always

Table 18.4.3. Adventitious regeneration from adult pear and quince explants.

Genotype	Mineral medium	Growth regulators	Other conditions tested	Maximum rate of regeneration	References
<i>Pyrus communis</i>					
Seckel, Louise Bonne	Nitsch and Nitsch	TDZ 3 µM + NAA 5.4 µM	Mother plants on BA (2.2 µM)	42–74%	Abu-Qaoud et al., 1991
Passe Crassane,	Modified Lepoivre	TDZ 2.5 µM + NAA 1 µM	Leaves from apical part of the shoot	90%	Chevreau and Leblay, 1993
Doyenné du Comice	Modified Lepoivre	TDZ 2.5 µM + NAA 1 µM	Agar/phytagel	47–95%	Chevreau et al., 1997a
Conférence, Doyenné du Comice, Passe Crassane, Seckel, Williams, Old Home, OHF333, Harrow Sweet			Cefotaxime + timentin		
<i>P. communis</i> var. <i>pyraster</i> L.	Quoirin and Lepoivre	BA 8.8 µM + NAA 1 µM	Leaves wounded by 3 cuts	40–64%	Caboni et al., 1999
			Cefotaxime		
<i>Pyrus pyrifolia</i>					
Chojuro, Kosui, Hosui, Nijisseiki, Shinchu, Okusankichi	B5 (Gamborg)	TDZ 1–5 µM + GA ₃ 0.25 µM	Older leaves	< 20%	Lane et al., 1998
			Incubation in dark		
<i>Pyrus syriaca</i>					
Endemic genotype	MS	TDZ 2 µM	Sucrose 0.15 M	76%	Shibli et al., 2000
			Leaves of intermediate age		
<i>Cydonia oblonga</i>					
East Malling A	MS–N6	TDZ 32 µM + NAA 0.3 µM	3 weeks of dark incubation	78%	Dolcet-Sanjuan et al., 1991
?	MS	TDZ 1.5 µM + NAA 2.5 µM	Sucrose > 3%	85%	Baker and Bhatia, 1993
			Leaves abaxial side down		

TDZ, thidiazuron.

successful. Spontaneous chromosome doubling occurred in a few cases and could not be controlled. Induced chromosome doubling was achieved in a reproducible way with 200–300 mM oryzalin. Shoots were immersed in liquid culture medium containing oryzalin and agitated for 48 h in darkness before placing them horizontally on semi-solid medium under light conditions. Homozygosity has been checked, using isozyme and microsatellite markers (Bouvier *et al.*, 2002). Acclimatization of doubled-haploid genotypes has been achieved only after *in vitro* micrografting on to a rootstock ('Old Home'). So far, 17 doubled-haploid clones from three cultivars ('Doyenné du Comice', 'Williams' and 'Harrow Sweet') are growing *in vitro*, six of them have been acclimatized in a greenhouse and four are growing in a nursery plot. This original homozygous material should offer new possibilities for genetic studies and breeding.

5.1.4. Protoplast isolation and culture

Regeneration from pear protoplasts was first reported by Ochatt and Caso (1986), using a wild pear (*P. communis* var. *pyraster*). This methodology was successfully extended to six pear genotypes in the late 1980s (Ochatt, 1993). In most cases, protoplasts were isolated enzymatically from the mesophyll of *in vitro*-grown leaves. Initial proliferation was obtained on medium free of ammonium ions (Ochatt and Patat-Ochatt, 1995). Plant regeneration from the protoplast-derived callus was always through organogenesis. However, no other team has reported successful regeneration from pear protoplasts. Preliminary results of quince protoplast isolation have been reported (D'Onofrio *et al.*, 1999), but no regeneration has been achieved.

5.2. Genetic manipulation

5.2.1. Mutation induction and somaclonal variation

Breeding objectives. Despite its potential for direct improvement of elite cultivars,

mutation induction has rarely been used for pear. Three mutants of Asian pear ('Gold Nijisseiki', 'Kotobuki Shinsui' and 'Osa Gold') have been released. They were obtained after chronic γ -irradiation of plants and were selected for increased resistance to black spot disease, caused by *A. alternata* (Masuda *et al.*, 1997; Yoshioka *et al.*, 1998). The main advantage of *in vitro* systems for mutation breeding is to decrease the risk of obtaining chimeric plants, either by using adventitious regeneration or by applying rapid cycles of micropropagation to separate mutated from non-mutated sectors. Very few data are available concerning the amount of somaclonal variation that can be recovered from pear tissue culture due to lack of efficient regeneration techniques from single cell origin, which frequently includes a period of callus growth prior to somatic embryo or adventitious bud regeneration. Somaclonal variation for traits of agronomical interest after adventitious bud regeneration has been reported at low frequencies (0.1–1.5%).

Protocol. Requirements for mutation induction include determination of the target tissue radiosensitivity and, when possible, development of an early and efficient screening test for the desired type of mutant. The radiosensitivity of pear microcuttings to X-rays was studied for 'Doyenné du Comice'. The median lethal dose (LD₅₀) was determined to be 30 Gy (Predieri *et al.*, 1986). The effect of γ - and ultraviolet (UV) irradiation of leaves on adventitious regeneration of four pear cultivars has also been studied. The LD₅₀ varied from 20 to 50 Gy for γ -irradiation and was about 125 J/m² for UV rays (Pinet-Leblay *et al.*, 1992). An *in vitro* screening assay for resistance to fire blight, based on the use of a pathogenicity mutant of *E. amylovora*, has been proposed by Pinet-Leblay *et al.* (1996) as a primary screen for resistance based upon hypersensitivity among mutagenized clones of susceptible pear cultivars. Preliminary screening for compact mutants has been proposed by Predieri and Govoni (1998), using increasing concentrations of BA in MS culture medium. Compact clones have been shown to have higher proliferation rates than the standard clones.

Somaclonal variation has been detected among regenerants produced by adventitious bud organogenesis from leaf (Caboni *et al.*, 2000) or leaf, stem and root explants (Viseur, 1990) and among quince regenerants from leaf explants (Dolcet-Sanjuan *et al.*, 1992). In all cases, no selection pressure was applied during the regeneration period. Ploidy level as determined by flow cytometry is an efficient way to detect tetraploid regenerants, which occur quite frequently in some genotypes (5–10% with 'Passe Crassane'). Some polymorphism was detected among pear regenerants in a random search using RAPD markers (Caboni *et al.*, 2000). Successive steps of *in vitro*, greenhouse and field selection tests were applied for fire blight (Viseur, 1990) and iron deficiency (Dolcet-Sanjuan *et al.*, 1992) tolerance.

Accomplishments. An extensive search for mutants has been conducted with six pear cultivars after γ -irradiation of microcuttings (Predieri and Zimmerman, 2001). About 1000 shoots of each cultivar were exposed to 35 Gy γ -irradiation dose. After three cycles of subculture, shoots were rooted and acclimatized. After 1 year in the nursery, more than 5000 self-rooted trees derived from irradiated shoots were planted in the field for assessment of pomological traits over a 6-year period. A sample of trees with variation in vegetative growth characters was further studied for 4 additional years. Variation involving productivity, period of bearing and fruit characters was observed at frequencies of from 1 to 3% and chimera behaviour was very rare. Several mutants with compact habit and high productivity will be released as new compact pear cultivars (Predieri, 2001). A number of mutants with increased sugar concentration or extended shelf-life are still under evaluation.

More than 300 clones have been regenerated from stem, root or leaf explants of three pear cultivars ('Durondeau', 'Conference' and 'Doyenné du Comice'). *In vitro* screening for fire blight resistance was performed by microcutting inoculation, and five clones with increased resistance were selected. After acclimatization, resistance was confirmed by greenhouse inoculations with *E.*

amylovora. Among the four somaclones of 'Durondeau', two were identified as tetraploids after chromosome counting. The two other diploids showed modifications of their branching habit, which could partly explain their increased level of resistance to fire blight (Viseur, 1990).

In addition, 1921 clones were regenerated from leaves of 'Quince A' and submitted to a two-step selection, in order to select variants with increased tolerance to iron deficiency. Nine clones survived the first screening *in vitro* on 0.1 mM FeSO_4 and two of the clones survived the second selection on medium without any iron. These two variants had higher chlorophyll content than the control and displayed a higher ability to reduce Fe(III) and to acidify the culture medium (Dolcet-Sanjuan *et al.*, 1992). This trait persisted under greenhouse conditions; however, initial field observations at a site with high soil pH and carbonate content, which limits Fe availability, indicated that the differences between the selected variants and the original quince genotype were not as pronounced and consistent as the differences detected in the greenhouse study (Bunnag *et al.*, 1996). More recently, quince 'BA29' somaclones were produced under high-pH selection pressure. Among 19 regenerated clones, two appeared to be more tolerant of Fe deficiency, as a result of enhanced ability to reduce pH during rooting and increased iron reduction efficiency *in vitro* and *in vivo* (Marino *et al.*, 2000).

The occurrence of somaclonal variation with respect to agronomical characters has been demonstrated, albeit at low frequency. The development of new regeneration techniques and the application of *in vitro* selection pressure for the trait of interest during the regeneration phase could probably increase the efficiency of this method.

5.2.2. Somatic hybridization

Somatic hybridization has been reported between wild pear (*P. communis* var. *pyraster*) and Colt cherry (*Prunus avium* $\times$ *P. pseudocerasus*), which are sexually incompatible rootstock genotypes (Ochatt *et al.*, 1989). The hybrids had the entire chromosome comple-

ments of the parents and intermediate leaf morphology and isozyme banding patterns. The use of these hybrids for pear improvement has not been recorded.

5.2.3. Genetic transformation

Breeding objectives. The main advantage of genetic transformation is to permit the targeted modification of one trait of an important cultivar without modifying its genetic background. After a useful transformant is selected, the new transgenic line can be maintained by vegetative propagation without the need of fixation through the sexual cycle. The length of time and the space requirement for complete evaluation of transformants are limiting factors that are as important as in the case of conventional breeding. Theoretically, all traits of interest for pear breeders are amenable to improvement via genetic transformation; however, to date genetic transformation of pear has focused on disease resistance, manipulation of fruit ripening and alteration of tree architecture and rooting.

Protocol. Pear is a natural host of *A. tumefaciens*, the causal agent of crown gall and natural vector of gene transfer (Lemoine and Michelesi, 1993). Transformation of pear is based on the co-culture of *in vitro* leaves with a disarmed *A. tumefaciens* strain carrying the gene(s) of interest in a binary vector. Most authors used disarmed strains such as derivatives of C58 or EHA; however, transformation of *P. betulaefolia* has been achieved using a C58 strain containing a wild-type Ti plasmid from strain AKE10, isolated from apple (Kaneyoshi *et al.*, 2001). Adventitious buds are regenerated in the presence of a selective agent (often kanamycin) in order to select for transformed cells carrying a kanamycin resistance gene. The process requires 1 year from the start of the experiment to acclimatization of transgenic plants in a greenhouse.

The first report of pear transformation concerned three European pear cultivars ('Passe Crassane', 'Conference' and 'Doyenné du Comice'), whose transformation rates varied from 1 to 40% (Mourgues

et al., 1996). Several other cultivars have been transformed, e.g. 'Vyzhnitsa' (Merkulov *et al.*, 1998), 'Beurre Bosc' (Bell *et al.*, 1999) and 'Bartlett' (Bommineni *et al.*, 2000), as well as rootstocks, e.g. *P. pyraster* (Caboni *et al.*, 1998), GP217 (Lebedev and Dolgov, 2000) and BP10030 (Zhu and Welander, 2000). A summary of the main features of pear transformation protocols is provided in Table 18.4.4. There have been no reports of transformation of *P. pyrifolia*, and only a preliminary report of transformation of quince genotype has appeared (Davidson *et al.*, 1998).

Accomplishments. Increased resistance to fire blight is the main objective of the pear genetic engineering programme at INRA in Angers. Two types of strategies have been adopted: (i) a direct lytic action on the pathogen with transgenes encoding lytic peptides of insect origin (attacin, cecropin) or lysozyme isolated from the T4 bacteriophage (Mourgues *et al.*, 1998); and (ii) a more specific inhibition of pathogenicity factors using a lactoferrin gene of bovine origin (for its possible competition with the bacterial siderophores) or a depolymerase gene (able to specifically degrade bacterial exopolysaccharides). Transformation experiments with these genes have produced a total of 81 transgenic clones from the cultivar 'Passe Crassane'. Semi-quantitative retro transcription (RT)-PCR revealed important differences among clones expressing the attacin E gene. These differences correlated very well with the differences of transgenic attacin E accumulation revealed by Western, i.e. protein, blot analysis. However, no clear correlation between transgene expression and resistance to fire blight as determined by *in vitro* inoculation has been established (Reynoird *et al.*, 1999). Among 16 transgenic diploid clones expressing the depolymerase gene, only two clones showed a slight reduction of fire blight symptoms in comparison to non-transformed plants. This partial resistance was correlated with a stronger expression of the transgene at transcriptional and translational levels. Very low depolymerase activity was detected in most transgenic clones (Malnoy *et al.*, 2002). Among nine

Table 18.4.4. Gene transfer to pear genotypes.

Genotype	Type of explant	<i>Agrobacterium</i> strain	Inoculation method	Selection	Rate of transformation (%)	References
Conference	Leaf	EHA	Scalpel cuts	Kanamycin 100 mg/l	1.2–42	Mourgues <i>et al.</i> , 1996
Passe Crassane					1.3	
Doyenné du Comice	Leaf	A281	Immersion	Kanamycin 50 mg/l	0.5	Merkulov <i>et al.</i> , 1998
Vyzhnitsa	Leaf	C58C1	–	Kanamycin	1.8	Caboni <i>et al.</i> , 1998
<i>P. communis</i> var. pyraister (6 genotypes)					–	
Beurre Bosc	Leaf	EHA101	Scalpel cuts	Kanamycin 80 mg/l for 4 weeks only	2–4	Bell <i>et al.</i> , 1999
Bartlett	Leaf	–	–	Kanamycin increasing from 15 to 50 mg/l	0.2–6.3	Bommineni <i>et al.</i> , 2000
BP10030	Internode				1.4–44	
OHF333	Leaf	C58C1	Forceps wound + immersion	Kanamycin decreasing from 100 to 50 mg/l	0.1	Zhu and Welsander, 2000
GP217	Leaf	CBE21 EHA	Immersion	Kanamycin 25 mg/l Hygromycin 5 mg/l	0	
					0.4–3	Lebedev and Dolgov, 2000
					6–11	
<i>P. betulaefolia</i> seedlings	Cotyledon	AKE10TC1	Immersion	Kanamycin 20–50 mg/l	2.5	Kaneyoshi <i>et al.</i> , 2001

transgenic clones expressing the lactoferrin gene, four expressed significantly fewer fire blight symptoms than the control. The increase of resistance was detected more strongly in the greenhouse than *in vitro*. A correlation with a mechanism of iron chelation was demonstrated by: (i) an increase of total iron content and ferric reductase activity in the transgenic plants; and (ii) an inhibitory activity of protein extracts from transgenic plants towards *E. amylovora* in conditions of iron deficiency (Malnoy *et al.*, 2003a). In order to obtain a more efficient and targeted expression of these transgenes, the search for inducible promoters has also been undertaken. Glucuronidase (GUS) fusions have been transformed into the cultivar 'Conference' to analyse the expression of several pathogen-inducible promoters from tobacco (Malnoy *et al.*, 2003b).

Transformation of the cultivar 'Bartlett' with another lytic peptide gene (*D5C1*) has been reported and its effect on a non-target organism (pear psylla) was reported (Puterka *et al.*, 2002).

A strategy to increase pear disease resistance which is currently being tested by a Russian team is the introduction of plant defensin genes from *Rhaphanus sativus* (Lebedev *et al.*, 2002a). The same group is also evaluating herbicide resistance of transgenic pear rootstocks transformed with the phosphotriester acetyl transferase (PAT) gene (Lebedev *et al.*, 2002b), and the modification of fruit taste conferred by the super-sweet gene thaumatin II (Lebedev *et al.*, 2002c).

The *rolC* gene from *Agrobacterium rhizogenes* has been introduced into the cultivar 'Beurre Bosc' under the control of its native promoter to induce a dwarfing effect (Bell *et al.*, 1999). The first observations on self-rooted or budded plants in the greenhouse indicated reduced height, number of nodes and leaf area of three transgenic clones. A similar approach has been pursued by Zhu and Welander (2000), who introduced the *rolB* gene into a dwarfing rootstock genotype (BP10030), which is very difficult to root. Recent results from cutting experiments in the greenhouse indicated a clear increase in rooting ability in the transgenic lines,

together with a modification of root morphology and shortened stem length (Zhu *et al.*, 2003).

The gene encoding S-adenosylmethionine hydrolase (*sam-k*) from bacteriophage T3 has been transferred into the cultivar 'Bartlett' in order to modify ethylene biosynthesis and improve postharvest quality and shelf-life (Bommineni *et al.*, 2000).

According to the Animal and Plant Health Inspection Service (APHIS) Field Test Releases Database (updated 16 July 2004), two groups have already released transgenic pears for field trials in the USA. The US Department of Agriculture (USDA)-Appalachian Fruit Research Station (ARS) in West Virginia has released transgenic pears carrying a cecropin gene for fire blight resistance and the *rolC* gene for dwarfing. Agritope, in Oregon, has released transgenic pears carrying the *sam-k* gene for delayed fruit ripening. No reports of field trials of transgenic pear have yet appeared in Europe.

5.3. Cryopreservation

Medium-term conservation of pear germplasm at low temperature was first proposed by Wanas *et al.* (1986). They reported a high survival rate of *P. communis* shoots after 18 months at 4°C, under light conditions. Wilkins *et al.* (1988) successfully tested the storage of *P. pashia* at 4 to 10°C under light for 12 months. Storage at low temperatures in continuous darkness was also effective for *P. pyrifolia* (Moriguchi *et al.*, 1990) and *P. syriaca* (Tahtamouni and Shibli, 1999).

Four *Pyrus* species were successfully cryopreserved using a cold-hardening period and a cryoprotectant mixture (Reed, 1990). Similar results were obtained with *P. communis* 'Williams' (Dereuddre *et al.*, 1990) and 'Beurre Hardy' (Scottez *et al.*, 1992) by embedding axillary shoot tips in alginate beads and dehydrating them at room temperature before freezing. Efficient cryopreservation techniques have also been developed for genotypes of *P. pyrifolia* (Niino and Sakai, 1992) and *P. syriaca* (Tahtamouni and Shibli, 1999).

Techniques for medium- and long-term storage of pear genetic resources are currently utilized at the National Clonal Germplasm Repository at Corvallis (USA), where 169 accessions of the primary field collection were kept as refrigerated *in vitro* cultures and more than 50 accessions were stored as apices in liquid nitrogen (Reed *et al.*, 1998a,b).

6. Conclusions

Selection among open-pollinated seedlings had been very successful in Europe between 1750 and 1850. Some of the most important *P. communis* cultivars were identified in this period. Programmes of controlled hybridization were later developed in Europe, North America, Japan and the southern hemisphere. Mutation induction, although efficient for some characters such as skin colour or compact growth, was never important for pear breeding. Production of European pear still relies upon a limited number of cultivars and, due to the relatively long juvenile period of pears, the rate of genetic improvement has been quite slow. Marketing of pears both within and among nations is characterized by limitations on the number of cultivars and slow acceptance of new cultivars. In contrast to peaches, which can be marketed as a commodity without regard to cultivar identity and recognition by consumers, the major pear cultivars are unique and are recognized by the consumer.

In this context, biotechnology can offer pear breeders new tools to increase the efficiency of hybridization and selection through the use of molecular markers, or to directly improve existing cultivars or elite genotypes through somaclonal variation or gene transfer. However, this field of study is still a very young one, with fewer than 20 years of biotechnology studies on pear (Chevreau and Skirvin, 1992). Adventitious regeneration techniques (Mehra and Jaidka, 1985; Ochatt and Caso, 1986; Predieri *et al.*, 1989) and molecular marker studies (Menendez and Daley, 1986) began to develop only in the late 1980s.

Genome analysis is still a very recent field of research for pear, and its importance should increase greatly in the next few years. Future development should include the establishment of a complete pear genetic map, taking into account the advantages of the synteny with the better known apple genome. Identification of markers of important agronomical characters will be a necessary step for the use of marker-assisted selection. The development of efficient techniques for differential gene expression studies and integrated analysis of genome expression should speed up the rate of elucidation of new pear gene functions (Fonseca *et al.*, 2004).

Much methodological progress has been made in the field of genetic engineering of pear in the last 10 years, and this has already resulted in the production of new plant genetic material, which is currently being evaluated. However, progress is still needed in order to adapt techniques to a larger range of varieties and rootstocks, and to develop selection techniques which avoid the need for antibiotic resistance transgenes. In the coming years, more diverse horticultural traits will certainly be amenable to genetic engineering, with increased knowledge of the genetic mechanisms underlying the traits of interest. However, before transgenic pears can be commercialized, several important issues need to be addressed. Risk assessment studies must be thoroughly conducted to evaluate the potential toxicity or allergenicity of the transgene products and the environmental consequences of an eventual gene flow to natural populations of wild *Pyrus*. More research efforts must be devoted to search for regulatory sequences enabling targeted and controlled expression of the transgenes, and stability of expression of the transgene throughout the tree life should be ensured. Practical use of transgenic pear varieties will not be possible without general public acceptance. Recent surveys indicate that public perception of the use of modern biotechnology for food production is much more negative in Europe than in the USA. Clear and balanced information is necessary to explain

how genetically modified varieties can help not only to increase the economic efficiency of pear production, but also to add beneficial traits of direct interest for consumers, without increasing environmental or health risks.

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18.5 Rubus spp. Cane Fruit

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1. Introduction

1.1. Botany and history

The genus *Rubus* (subfamily *Rosoideae*) (Bailey, 1949) includes other horticulturally important crops such as strawberries and roses. The *Rubus* species are collectively referred to as brambles and include blackberries, dewberries and raspberries. The genus *Rubus* is prominent in North America, comprising > 400 species (Bailey, 1949) in 12 subgenera (Focke, 1910–1914; Jennings, 1988; Table 18.5.1). Dr Kim Hummer (Curator, US Department of Agriculture (USDA) *Rubus* germplasm collection, Corvallis, Oregon, USA) has prepared a list of the most important *Rubus* species (Table 18.5.2). Among the

three largest subgenera, *Eubatus* (blackberries) and *Idaeobatus* (raspberries) are common in cultivation (Watson, 1958; Robertson, 1974), while *Malachobatus* has no obvious pomological value. Commercial *Rubus* crops include red (*R. idaeus* L.), black (*R. occidentalis*) and purple (hybrid between red and black) raspberries, blackberries (*Rubus* spp. and hybrids), cloudberrries (*R. chamaemorus*) and Andean (*Rubus* spp.) blackberries (Thompson, 1997).

The fruit of a bramble is an aggregate and develops from a flower with multiple pistils attached to a single receptacle. Each pistil develops into a drupelet, and each fruit has many drupelets. Raspberries are distinguished from blackberries by how they pick from the plant. The raspberry receptacle

Table 18.5.1. Focke's (1910–1914) 12 subgenera of *Rubus* as reported by Jennings (1988).

Subgenus 1	<i>Chamaemorus</i>	1 species	<i>R. chamaemorus</i> L., the cloudberry
Subgenus 2	<i>Dalibarda</i>	5 species	No pomological value
Subgenus 3	<i>Chamaebatus</i>	5 species	No pomological value
Subgenus 4	<i>Comaropsis</i>	2 species	No pomological value
Subgenus 5	<i>Cylactis</i>	14 species	Arctic berries including <i>R. arcticus stellatus</i> Sm.
Subgenus 6	<i>Orobatus</i>	19 species	Probably related to subgenus 1 (<i>Chamaemorus</i>)
Subgenus 7	<i>Dalibardastrum</i>	4 species	No pomological value (Asia)
Subgenus 8	<i>Malachobatus</i>	114 species	No pomological value; some are ornamentals
Subgenus 9	<i>Anoplobatus</i>	6 species	(Flowering raspberries)
Subgenus 10	<i>Idaeobatus</i>	200 species	Raspberries
Subgenus 11	<i>Lampobatus</i>	10 species	No pomological value
Subgenus 12	<i>Eubatus</i>	Very large number of species	Blackberries

Table 18.5.2. *Rubus* species, subgenera, sections and common names (from Wiersema, 2002).

Species name	Subgenus	Section	Common name
<i>R. allegheniensis</i> Porter	<i>Rubus</i>	<i>Allegheniensis</i>	Allegheny blackberry
<i>R. alumnus</i> L.H. Bailey	<i>Rubus</i>	<i>Allegheniensis</i>	Kittatinny blackberry
<i>R. arcticus</i> L.	<i>Cyclactis</i>		Arctic bramble
			Arctic raspberry
			Nectarberry
<i>R. arcticus</i> L. subsp. <i>notho</i>	<i>Cyclactis</i>		Arctic bramble
<i>Stellarcticus</i> G. Larss.			Arctic raspberry
<i>R. arcticus</i> L. subsp. <i>stellatus</i> (Sm.) B. Boivin	<i>Cyclactis</i>		Alaskan raspberry
			Nangoonberry
<i>R. arcticus</i> L. subsp. <i>arcticus</i>	<i>Cyclactis</i>		Arctic bramble
			Arctic raspberry
<i>R. argutus</i> Link	<i>Rubus</i>	<i>Arguti</i>	Prickly Florida blackberry
<i>R. bambusarum</i> Focke	<i>Malachobatus</i>		
<i>R. biflorus</i> Buch.-Ham. ex Sm.	<i>Idaeobatus</i>		Raspberry
<i>R. bogotensis</i> Kunth	<i>Lampobatus</i>		Mora de cerro
<i>R. caesius</i> L.	<i>Rubus</i>	<i>Caesii</i>	European dewberry
<i>R. canadensis</i> L.	<i>Rubus</i>	<i>Canadenses</i>	Canadian blackberry
<i>R. chamaemorus</i> L.	<i>Chamaemorus</i>		Cloudberry
<i>R. chingii</i> Hu	<i>Idaeobatus</i>		Chinese raspberry
<i>R. chroosepalus</i> Focke	<i>Malachobatus</i>		
<i>R. cissburiensis</i> W.C. Barton & Ridd.	<i>Rubus</i>	<i>Rubus</i>	Blackberry
<i>R. cissoides</i> A. Cunn.	<i>Micranthobatus</i>		
<i>R. cockburnianus</i> Hemsl.	<i>Idaeobatus</i>		Raspberry
<i>R. corchorifolius</i> L. f.	<i>Idaeobatus</i>		Raspberry
<i>R. coreanus</i> Miq.	<i>Idaeobatus</i>		Raspberry
<i>R. crataegifolius</i> Bunge	<i>Idaeobatus</i>		Raspberry
<i>R. ellipticus</i> Sm.	<i>Idaeobatus</i>		Yellow Himalayan raspberry
<i>R. eustephanos</i> Focke ex Diels	<i>Idaeobatus</i>		Raspberry
<i>R. fruticosus</i> auct. group	<i>Rubus</i>	<i>Rubus</i>	Blackberry
<i>R. geoides</i> Sm.	<i>Comaropsis</i>		
<i>R. glaucus</i> Benth	<i>Lampobatus</i>		Andean blackberry
<i>R. grabowskii</i> Weihe ex Günther et al.	<i>Rubus</i>	<i>Rubus</i>	Blackberry
<i>R. hawaiiensis</i> A. Gray	<i>Idaeobatus</i>		Hawaiian raspberry
<i>R. hayata-koidzumii</i> Naruh.	<i>Chamaebatus</i>		Yü-shan raspberry
<i>R. henryi</i> Hemsl. & Kuntze	<i>Malachobatus</i>		
<i>R. hirsutus</i> Thunb.	<i>Idaeobatus</i>		Raspberry
<i>R. humulifolius</i> C.A. Mey	<i>Cyclactis</i>		
<i>R. hypargyus</i> Edgew. var. <i>niveus</i> H. Hara	<i>Idaeobatus</i>		
<i>R. ichangensis</i> Hemsl. & Kuntze	<i>Malachobatus</i>		
<i>R. idaeus</i> L.	<i>Idaeobatus</i>		European red raspberry
<i>R. illecebrosus</i> Focke	<i>Idaeobatus</i>		Strawberry raspberry
<i>R. inominatus</i> S. Moore	<i>Idaeobatus</i>		Raspberry
<i>R. lambertianus</i> Ser.	<i>Malachobatus</i>		
<i>R. lasiococcus</i> A. Gray	<i>Cyclactis</i>		
<i>R. lasiostylus</i> Focke	<i>Idaeobatus</i>		
<i>R. leucodermis</i> Douglas ex Torr. & A. Gray	<i>Idaeobatus</i>		Western black raspberry
<i>R. loganobaccus</i> L.H. Bailey	<i>Rubus</i>	<i>Ursini</i>	Loganberry, boysenberry
<i>R. megalococcus</i> Focke	<i>Lampobatus</i>		

Continued

Table 18.5.2. *Continued.*

Species name	Subgenus	Section	Common name
<i>R. microphyllus</i> L. f. var. <i>subcrataegifolius</i> (H. Lev. & Vaniot) Ohwi	<i>Idaeobatus</i>		Raspberry
<i>R. moluccanus</i> L.	<i>Malachobatus</i>		Raspberry
<i>R. neomexicanus</i> A. Gray	<i>Anoplobatus</i>		New Mexico raspberry
<i>R. nepalensis</i> (Hook. f.) Kuntze	<i>Dalibardastrum</i>		
<i>R. nessensis</i> Hall	<i>Rubus</i>	<i>Rubus</i>	Blackberry
<i>R. niveus</i> Thunb.	<i>Idaeobatus</i>		Mysore raspberry
<i>R. occidentalis</i> L.	<i>Idaeobatus</i>		Eastern black raspberry
			Blackcap
<i>R. odoratus</i> L.	<i>Anoplobatus</i>		Flowering raspberry
<i>R. parviflorus</i> Nutt.	<i>Anoplobatus</i>		Salmonberry
			Thimbleberry
<i>R. parvifolius</i> L.	<i>Idaeobatus</i>		Japanese raspberry
			Trailing raspberry
<i>R. parvus</i> Buchanan	<i>Micranthobatus</i>		
<i>R. pedatus</i> Sm.	<i>Cyclactis</i>		Mossberry
<i>R. phoenicolasius</i> Maxim.	<i>Idaeobatus</i>		Wineberry
<i>R. pinfaensis</i> H. Lev. & Vaniot	<i>Idaeobatus</i>		
<i>R. plicatus</i> Weihe & Nees	<i>Rubus</i>	<i>Rubus</i>	Blackberry
<i>R. procerus</i> P.J. Mull. ex Boulay	<i>Rubus</i>	<i>Rubus</i>	Himalayan blackberry
<i>R. rolfei</i> S. Vidal	<i>Malachobatus</i>		
<i>R. rosaceus</i> Weihe	<i>Rubus</i>	<i>Rubus</i>	Blackberry
<i>R. roseus</i> Poir	<i>Lampobatus</i>		Mora silvestre
<i>R. rosifolius</i> Sm.	<i>Idaeobatus</i>		Mauritius raspberry
<i>R. sachalinensis</i> H. Lev.	<i>Idaeobatus</i>		Raspberry
<i>R. saxatilis</i> L.	<i>Cylactis</i>		
<i>R. shankii</i> Standl. & L.O. Williams	<i>Lampobatus</i>		Honduran blackberry
<i>R. spectabilis</i> Pursh	<i>Idaeobatus</i>		Salmonberry
<i>R. squarrosus</i> Fritsch	<i>Micranthobatus</i>		
<i>R. strigosus</i> Michx.	<i>Idaeobatus</i>		American red raspberry
<i>R. sumatranus</i> Miq.	<i>Idaeobatus</i>		
<i>R. tephrodes</i> Hance	<i>Malachobatus</i>		
<i>R. trifidus</i> Thunb.	<i>Idaeobatus</i>		
<i>R. ulmifolius</i> Schott	<i>Rubus</i>	<i>Rubus</i>	Blackberry
			Zarzamora
<i>R. ursinus</i> Cham. & Schltdl.	<i>Rubus</i>	<i>Ursini</i>	California blackberry
			Pacific blackberry
<i>R. vernus</i> Focke	<i>Idaeobatus</i>		Japanese salmonberry

This list was prepared by Dr Kim Hummer, curator, USDA *Rubus* germplasm collection, Corvallis, Oregon, USA.

separates from the fruit and remains on the plant (free receptacle); the receptacle of a blackberry fruit separates from the plant and adheres to the fruit.

Although some *Rubus* spp. have perennial stems, most economically important brambles have perennial roots and crowns that produce flowering structures (canes), which live for 2 years (Moore and Skirvin, 1990).

New canes are produced from either the crown or the roots (root suckers). The new canes are generally vegetative and are referred to as primocanes. After the primocanes have experienced a winter period (vernalization) they become reproductive and are referred to as floricanes. Floricanes produce flowers and fruit and then die. Most growers remove the spent floricanes to make

more room for primocanes to grow for the next season's crop. The primocanes of some types of brambles are able to produce flowers without vernalization. Plants of this sort are called primocane flowering types. The most famous of these is the 'Heritage' raspberry, which is well adapted to most of the eastern USA and southern Canada. The canes of 'Heritage' begin to flower when the canes reach approx. their 40th node. They will continue to flower and fruit until a hard freeze. In the following spring, they will begin to flower again near where they stopped in the autumn and flower for many more nodes until they die and are replaced by primocanes. Many growers have found that 'Heritage' can be mowed to the ground in late winter. The spring crop is lost by mowing, but the primocanes will produce a good crop in the autumn with little or no hand labour (Skirvin and Otterbacher, 1979).

Brambles are grown in many parts of the world, but they grow best and provide the most economic returns when cultivated in areas with mild winters and long dry summers. Ideally, the plants should receive adequate moisture either by natural rainfall or by irrigation. Major regions of bramble production in North America include the Pacific North-West (Oregon, Washington and British Columbia), California and parts of Texas and Arkansas, as well as regions of lake temperature moderation in New York, Michigan, Pennsylvania and Ohio. In Europe brambles are grown extensively in the UK, especially Scotland. The European Union (EU) produces large quantities of brambles for both fresh and processing use. Poland and other central European countries produce large quantities of fruit for processing into jams, jellies and juices. Brambles are grown extensively in Chile, Argentina and Guatemala for export to northern hemisphere markets during their winter season. Large quantities of brambles are grown in New Zealand for processing.

The members of the genus *Rubus* can be difficult to classify into distinct species (Robertson, 1974). Members of the *Malachobatus* come from South-east Asia, Japan, Australia and Madagascar (Jennings, 1988). The *Idaeobatus* has a northerly distrib-

ution, mainly in Asia, east and southern Africa, Europe and North America. The *Eubatus* is very complex and has sections in South America, Europe and North America. The problem of distinguishing plants is further aggravated by the fact that many species and cultivars of diverse backgrounds have been extensively hybridized.

Apomixis affects *Rubus* identification because offspring of apomictic plants can be clones of the mother plant and offspring of such plants are either completely or partially clones of the mother plant (a mixture of sexual and asexual offspring). Thus, it is not unknown for particular regions to be populated with a single clone of plants. For instance, the most common weedy blackberry found in the Pacific Northwestern USA is the 'Himalaya' blackberry, *R. armeniacus* Focke (= *R. procerus*; *R. discolor*), an apomictic species introduced from Europe in 1885 by Luther Burbank (Hummer, 1996). Seeds of 'Himalaya' are apomictic and birds have spread the plant extensively. One of its relatives, *R. fruticosus* L., has been declared a noxious weed by the USDA APHIS. This 'species' cannot be legally brought into the USA from foreign countries without a permit (Hummer, 1996).

The most economically important brambles are the raspberries. More than 200 species have been recognized (Jennings, 1988). The red (*R. idaeus* L) and black raspberries (*R. occidentalis*) are the best known types grown in Europe, Asia and North America. Most diploid raspberries reproduce sexually; polyploids tend to be apomictic and/or sexual.

1.2. Breeding and genetics

Rubus breeding is hampered by several genetic problems, including polyploidy, apomixis, pollen incompatibility and poor seed germination. Most blackberry cultivars are tetraploid, but ploidy levels range from diploid to $2n = 14x = 98$ or possibly $2n = 18x = 126$ (Thompson, 1995a,b). Polyploidy can complicate a breeding programme when two parents of different ploidy levels are crossed. Pairing of chromosomes in such a cross is

not uniform, and it can be difficult to obtain fertile offspring. Meng (1998) has reported an efficient method to determine DNA content and basic ploidy level in *Rubus* using flow cytometry.

Facultative apomixis complicates blackberry breeding. There are two forms of apomixis, adventitious embryony and gametophytic apomixis. The ability to distinguish apomicts from sexually derived seedlings is difficult and has impeded breeding of all apomictic *Rubus* spp. Comparing DNA banding patterns of hybrids and suspected apomicts to parents could distinguish the two types of offspring. Darrow and Waldo (1933) reported that crosses made with *R. laciniatus* 'Thornless Evergreen' as the female parent produced 87.3% apomictic offspring. Hall *et al.* (1986a) described crosses with 'Thornless Evergreen' as producing 93.9% apomictically derived offspring. The small number of hybrid offspring are difficult to distinguish from apomictically derived offspring by morphological characters alone (Kraft and Nybom, 1995).

Repeated breeding with particular parental stock plants leads to a lack of genetic diversity and can result in inbreeding depression. Poor fruit set coupled with apomixis further limits the number of hybrid progeny. Identification of closely related individuals and cultivars for use as parents is further restrained by the fact that some botanical characters and horticultural properties are influenced by environmental factors. Peiterson (1921), for example, claimed that blackberry plants grown in the shade have fewer thorns than those grown in the sun. These factors are compounded by the occasional mislabelling of accessions in germplasm collections, and can affect the results of a specific plant cross.

1.2.1. Breeding objectives

Bramble breeding is traditionally accomplished by hybridization between cultivars and/or species with desirable characteristics for multiple generations. Each cycle of hybridization involves a cycle of field observation. Breeding goals vary from region to region, but there are several traits that are

considered important. These include: (i) adaptation to mechanical harvesting; (ii) increased cold hardiness; (iii) resistance to grey mould caused by *Botrytis cinerea*, raspberry bushy dwarf virus (RBDV) and double blossom or rosette caused by *Cercospora rubi*, which limits blackberry production in the south-eastern USA (Gupton, 1999); (iv) thornlessness; (v) large fruit size; (vi) resistance to raspberry aphids *Amphorophora idaei* and *Amphorophora agathonica*; (vii) low chilling requirement for tropical and subtropical regions; (viii) small seed size; (ix) improved flavour, colour and processing quality (Hull, 1968; Ourecky, 1975; Moore, 1984; Hall, 1990; Hokanson, 2001); and (x) extended shelf-life. There is an increasing interest in primocane fruit production in blackberries and raspberries, which might overcome the low cold hardiness problems generally associated with the thornless character (Hokanson, 2001).

1.2.2. Breeding accomplishments

Bramble breeders have successfully produced plants that vary in their growth habit, e.g. erect, semi-erect and procumbent, resistance to bacterial, fungal and viral diseases, ability to flower on primocanes in raspberry and blackberries, thornlessness and fruit firmness and quality (Daubeney, 1996). According to Martin (2002) pests and pathogens of raspberries have evolved rapidly and have overcome host resistance in new cultivars, e.g. RBDV (Barbara *et al.*, 1984) and raspberry aphids *A. idaei* and *A. agathonica* (Daubeney, 1996).

2. Molecular Genetics

2.1. Gene cloning

Some bramble genes have been identified and partially or fully sequenced. These are listed at <http://www.ncbi.nlm.nih.gov/dbEST> home pages coordinated by the National Center for Biotechnology Information for the National Institutes of Health (NIH).

Jones, C.S. *et al.* (1999) have identified 20 genes that are associated with fruit ripen-

ing in 'Glen Clova' red raspberry. Many of these genes are implicated in ethylene biosynthesis and cell wall hydrolysis. Medina-Escobar *et al.* (1998) identified a low-molecular-weight heat-shock protein that is present at high levels in achenes during seed development and in the receptacle during fruit ripening.

2.2. Molecular markers

Molecular markers for use in *Rubus* include isozymes and DNA-based markers such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs), sequence-characterized amplified regions (SCARs), microsatellites and DNA sequences. These markers have been used for cultivar identification, identification of somaclonal mutations, parentage testing, identification of hybrids, taxonomy studies, studies of genotypic distribution in wild *Rubus* populations, studies of the amount of genetic variation in wild *Rubus* populations, disease diagnostics, genome mapping and marker-assisted selection. These topics have been comprehensively reviewed by Antonius-Klemola (1999) and Hokanson (2001).

2.2.1. Taxonomic relationships

Non-DNA-based markers. Haskell and Garrie (1966) utilized two-way paper chromatography to distinguish seven wild and cultivated raspberry (*R. idaeus*) varieties by genotype according to their flavonoid spots and spotting patterns. Little difference was detected among some cultivars and they concluded that this procedure was not sensitive enough to identify cultivars. Cousineau and Donnelly (1989) improved raspberry cultivar identification by isozyme analysis. They assayed plant extracts from 20 different *Rubus* cultivars for the presence of specific enzyme activity. Five enzymes were successfully used to characterize 14 out of 20 plants; however, isozyme analysis lacks the absolute specificity needed for cultivar identification.

DNA-based markers. To detect interspecific and intraspecific genetic variation among four *Rubus* spp., Nybom *et al.* (1990) hybridized an M13 bacteriophage probe to DNA of 14 different *Rubus* plants. They could detect variable length repetitive sequences in the plant genome, and demonstrated high genetic variation among the selected taxa. They suggested that this procedure could reveal genetic differences among closely related species or cultivars. Antonius and Nybom (1994) later used the M13 probe to assess genotypic distribution among 24 red raspberry (*R. idaeus*) plants in natural geographic settings. The probe produced unique DNA fingerprint patterns, which suggested that plants with a high genetic similarity were either apomicts or clones while those of low similarity resulted from outcrossing. Kraft and Nybom (1995) later used the M13 probe to estimate levels of apomixis and outcrossing among *Rubus* from Denmark, Germany and Sweden.

Kraft *et al.* (1996) used a radioactively labelled minisatellite probe to demonstrate that new *Rubus* species could evolve by interspecific hybridization and that stabilization of these species could occur through apomictic seed production. Morphologically similar species could be distinguished with the presence of minisatellite segments for each clone. The fingerprints of outcrossing species varied considerably, whereas vegetative and apomictic propagation of a clone resulted in identical DNA fingerprints.

Waugh *et al.* (1990) investigated variation in 20 diverse *Rubus* genotypes by the hybridization of chloroplast DNA (cpDNA) sequence probes derived from barley (*Hordeum*) and pea (*Pisum*). The probe hybridization data were employed to construct a phylogenetic tree to estimate the specific relationships among genotypes. The cpDNA sequence provided information that was unavailable through conventional morphological examination. Howarth *et al.* (1997) studied taxonomic relatedness by analysis of sequence variations in the cpDNA of *Rubus* subgenus *Idaeobatus* from the Hawaiian islands. Sequence analysis of the *ndhF* gene was successful in determining phylogenetic relationships within the sub-

genus *Idaeobatus*. The gene encodes a relatively conserved subunit of the nicotinamide adenine dinucleotide (NADH) dehydrogenase enzyme. Most recently Alice and Campbell (1999) analysed the base sequence of a nuclear ribosomal DNA internal transcribed spacer (ITS) region. Like Waugh *et al.* (1990) and Howarth *et al.* (1997), Alice and Campbell (1999) constructed a phylogenetic tree displaying the lineage of 57 *Rubus* taxa from 20 *Rubus* species. Non-nuclear DNA (ribosomal and chloroplast) investigations have been in agreement with previous taxonomic studies and are considered important resources for determining genetic relatedness (Alice *et al.*, 1997).

Parent and Pagé (1992) used alkaline phosphatase-labelled probe hybridization and chemiluminescence detection. Unique DNA fingerprint patterns were obtained from 13 red raspberry and two purple raspberry plants by non-radioactive DNA fingerprinting. They considered their technique reliable enough to identify unique raspberry cultivars.

Graham *et al.* (1994) used the polymerase chain reaction (PCR) with arbitrary primers to create specific banding patterns for red raspberry cultivars. They were able to distinguish among ten red raspberry cultivars by producing unique banding patterns using ten random primers. The general relationship among cultivars was estimated and the procedure was sensitive enough to distinguish closely related vegetatively propagated cultivars. Graham and McNicol (1995) used RAPD markers on 13 different *Rubus* species (24 different accessions) to assess the degree of similarity among species. RAPDs were in general agreement with existing knowledge of the origins of the germplasm. The authors reported their efforts to identify genomic changes between 'Williamette' (thorny raspberry) and 'Thornless Williamette', whose thornlessness is believed to result from a single mutation, using ten RAPD primers. Unfortunately, genetic difference between the two types could not be detected using RAPD markers.

Graham *et al.* (1997) used five random primers to screen eight *Rubus* genotypes

including raspberry, blackberry and hybrid-blackberry (red raspberry × blackberry) plants for their ability to regenerate shoots on various tissue culture media. Correlations were made between their genetic similarity and their ability to regenerate. Three thornless blackberries were included in this study, 'Chester Thornless', 'Hull Thornless' and 'Loch Ness'. With five random primers, 'Chester Thornless' and 'Hull Thornless' were determined to be 91% genetically similar. This is not surprising since they originated from the same cross and are sister seedlings (Galletta *et al.*, 1998).

RAPDs have also been used to distinguish 11 different thornless blackberry cultivars (Coyner, 2000). Results were analysed with clustering statistics. *R. laciniatus*-type blackberries segregated from other thornless types into a single tight cluster. 'Austin Thornless', 'Merton Thornless' types and 'Whitford Thornless' combined to form one large cluster, which was further divided into two subgroups, one containing only 'Merton Thornless' types and the other containing 'Austin Thornless', several 'Merton Thornless' types and 'Whitford Thornless'. 'Austin Thornless' and 'Whitford Thornless' are morphologically distinct and possess diverse chromosomal arrangements; however, RAPD marker data indicated that these blackberries are more genetically similar than expected.

One hundred and thirteen cultivar-specific RAPD fragments, generated from 75 unique primers, could be used to identify an accessioned plant without phenotypic reference. Two of these primers can distinguish among 'Everthornless', 'Thornless Evergreen' and 'Evergreen' (Coyner, 2000). These *R. laciniatus* plants are believed to be genotypically identical except for a single mutation which led to the thornless condition (McPheeters and Skirvin, 1995, 2000; Coyner, 2000).

2.3. Genome mapping

The task of mapping the *Rubus* genome has been proposed but remains largely unexplored. An exception is a report made by

Lim *et al.* (1998), who examined the chromosomes of a blackberry, a raspberry and their allopolyploid hybrids using genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH). With GISH they were able to distinguish raspberry and blackberry chromosomes as well as blackberry chromosomes carrying raspberry translocations.

3. Micropropagation

Most commercial brambles are now routinely maintained, propagated and sold as disease-indexed plants using *in vitro* methods. Broome and Zimmerman (1978) and Harper (1978) first reported proliferation of *Rubus* shoots *in vitro*. Procedures for reliable rooting and acclimatization of *in vitro* plants have also been described (Broome and Zimmerman, 1978; James, 1979; Skirvin *et al.* 1981; Deng and Donnelly, 1993). There can be problems at various stages of micropropagation, including losses during the acclimatization phase (Williamson *et al.*, 1998). Sobczykiewicz (1987) described the methods used to mass propagate raspberry plants using meristem culture. Micropropagation of *Rubus* has been reviewed previously (Skirvin, 1981; McPheeters *et al.*, 1988; Pelto and Clark, 2000a,b). Several *Rubus* species have responded well on semi-solid Murashige and Skoog (1962) (MS) medium supplemented with 8.9 μ M benzyladenine (BA), 0.54 μ M naphthaleneacetic acid (NAA) and the Staba (1969) vitamin mixture (R. Skirvin, unpublished data). Cultures have been grown at 25°C under a 16 h photoperiod with light supplied by cool white fluorescent tubes. Bramble cell lines have been used for biochemical analyses (Elder *et al.*, 1977). Chang and Reed (1999) have also reported the *in vitro* growth of several hundred *Rubus* species and accessions, and have described a protocol in depth.

4. Disease-indexed Plants

A method has been described for eliminating virus by thermotherapy (Lankes, 1995). The procedure involves incubation of virus-

infected plants at relatively high temperature (c. 36–38°C) for approx. 3 weeks. At the end of this period, meristem tips (c. 0.5 mm) are excised and explanted on to semi-solid plant growth medium until plantlets are large enough to be indexed by enzyme-linked immunosorbent assay (ELISA) or PCR.

5. Somatic Cell Genetics

5.1. Regeneration

Although biotechnological procedures can be used to explore variation and characterize reproductive, morphological and enzymatic characters of plants directly, the lack of a reliable system for regenerating shoots has impeded progress in the development of genetically engineered brambles (Hokanson, 2001).

5.1.1. Somatic embryogenesis

There have been three published accounts of somatic embryogenesis of *Rubus*, all involving cotyledon explants (Fiola and Swartz, 1986; Cantoni *et al.*, 1993; Gingas and Stokes, 1993). Unless they arose from an apomictic parent, such explants should not be expected to be clonal and consequently may have little direct importance for bramble cultivar improvement. The development of a reliable method for induction of embryogenic cultures of elite *Rubus* would be invaluable for transformation.

5.1.2. Organogenesis

Induction. Regenerants obtained by organogenesis are usually induced on MS medium enriched with cytokinin and auxin (Swartz and Stover, 1996). Blackberries and raspberries have been regenerated from leaves, cotyledons and internodal stem segments (Hall *et al.*, 1986b). DeFaria *et al.* (1997) reported optimal regeneration and transformation rates for 'Comet' red raspberry when 10 mm leaf discs were used as explants and plants were subcultured at 4-week intervals. Leaf orientation on the medium can affect organogenesis (McNicol and Graham, 1990).

R. laciniatus displays site-specific regeneration on leaf explants (Ke *et al.*, 1988a,b; Norton, 1994). Shoots that regenerate from leaf explants almost invariably form at the leaf-petiole junction. Cytohistological studies (Ke *et al.*, 1989) showed that organogenesis is initiated in phloem parenchyma cells or cells surrounding the vascular bundles. Cells became swollen, with accumulations of starch after 5 days in culture. Meristematic masses formed after 10 days, elongating into leaf-like tissue. Adventitious buds can form from the epidermis of this leaf-like tissue.

Genotypic differences are the most critical factors for organogenesis (McNicol and Graham, 1990; Reed, 1990; Owens *et al.*, 1992; Graham *et al.*, 1997). Thidiazuron (TDZ) is more effective than other cytokinins (Fiola *et al.*, 1990; McNicol and Graham, 1990; Norton, 1994). Millan-Mendoza and Graham (1999) observed that the synthetic cytokinin *N*-(2-chloro-4-pyridyl)-*N*-phenylurea (CPPU) improved frequency of organogenesis in raspberry, blackberry and a blackberry × raspberry hybrid. For organogenesis of blackberry, cytokinin is generally used in combination with an auxin such as indolebutyric acid (IBA), NAA or 2,4-dichlorophenoxyacetic acid (Ke *et al.*, 1988a,b; Norton, 1994; Norton and Skirvin, 1997). Environmental conditions also affect organogenesis. Explants are usually cultured in the dark from 1 to several weeks before being transferred to light conditions (Fiola *et al.*, 1990; McNicol and Graham, 1990; Norton, 1994; Meng *et al.*, 2004). Turk *et al.* (1993) found that 20°C was superior to 25°C for regeneration.

Development. Shoots that originate from axillary buds and adventitious meristems are rooted on medium that includes either 4.9–9.8 µM IBA or 5.4–10.7 µM NAA (Skirvin, 1981; McPheeters *et al.*, 1988). Rooting of *in vitro* shoots as cuttings has not been particularly successful. Shoots with roots can be transferred to *ex vitro* conditions and acclimatized under intermittent mist in the shade. After the emergence of the first leaf, the plants can be removed from acclimatization conditions, and will produce a crop 2 years later.

5.1.3. Triploid recovery

Bernasconi *et al.* (1993) established endosperm-derived callus lines of blackberry; however, regeneration was not observed.

5.1.4. Protoplast isolation and culture

Protoplasts from various *Rubus* species have been isolated (Infante *et al.*, 1993) and utilized for physiological investigations (Dunand *et al.*, 2000; Nita-Lazar *et al.*, 2000). Mazzetti *et al.* (1999) attempted to fuse protoplasts of 'Autumn Bliss' raspberry with 'Hull Thornless' blackberry. Fusion products were identified using RAPD markers. Callus lines were produced but regeneration did not occur.

5.2. Genetic manipulation

5.2.1. Mutation induction and somaclonal variation

Although the origin of somaclonal variation is not well understood, there are genetic mechanisms associated with it that have been studied in some detail (Skirvin, 1978; Larkin *et al.*, 1989; Skirvin *et al.*, 2000). An estimate of 1–3% total variation per generation cycle may be conservative; others have estimated somaclonal variation to be greater than 10% for each cycle (Larkin *et al.*, 1989). Somaclonal variation has been observed among both blackberries (McPheeters and Skirvin, 1983, 1989; Norton, 1994; Norton and Skirvin, 1997) and raspberries (Hoepfner *et al.*, 1996). The amount of variability can be genotype-specific (Skirvin, 1978). Exploitation of somaclonal variation is especially applicable to older vegetatively propagated cultivars or clones, which could be expected to have accumulated large numbers of mutant somatic cells after decades or centuries of vegetative propagation (Skirvin *et al.*, 1994). Somaclonal variation has been used to produce unique thornless brambles.

Hall *et al.* (1986b,c) released a tissue culture-derived bramble cultivar, 'Lincoln Logan'. They separated the histogenic layers of chimeral 'Thornless Logan' to form a pure thornless type (Hall *et al.*, 1986c). The

so-called thorns (spines) of brambles are of epidermal origin. Therefore, a thornless plant is really a hairless plant. 'Lincoln Logan' was believed to be derived from epidermal cells and therefore genetically pure thornless. The root suckers were thornless and indicated that the chimera had segregated. 'Lincoln Logan' is identical to 'Logan' (aka 'Loganberry') (Waldo and Hartman, 1946; Hull, 1968). Rosati *et al.* (1988) found that the thornlessness gene (S_{tl}) of non-chimeral 'Loganberry' is dominant. The unique pure thornless plant has been used as a parent for bramble improvement in the Pacific coastal region of the USA (Meng, 1998).

'Everthornless' is a genetically pure form of 'Thornless Evergreen' that was obtained via somaclonal variation (McPheeters and Skirvin, 1995; Plant Patent No. 9407). 'Thornless Evergreen' is a thornless mutant of 'Evergreen' (*R. laciniatus*), which grows wild in the Pacific North-west. 'Thornless Evergreen' is a periclinal chimera, in which the epidermis is composed of cells carrying a dominant gene (S_{te}) for thornlessness (Hall *et al.*, 1986a), while the internal tissues are composed of cells having a thorny genotype (Darrow, 1931). McPheeters and Skirvin (1983) separated the genotypes in this chimeral blackberry by rapid *in vitro* propagation. In the case of 'Thornless Evergreen' blackberry, one would expect to find both thorny and thornless plants in approximately equal quantities among the segregants; however, all regenerants are thornless. Some regenerants (c. 50%) were vigorous (normal) like the parent, whereas the other half (dwarf) were weak and dwarfed. The dwarf plants yielded thornless root suckers, indicating that they had been derived adventitiously from the epidermis and were pure thornless. The vigorous plants produced thorny root suckers, indicating they were still chimeral and identical to the parent. No thorny plants were observed (McPheeters and Skirvin, 1983). Although no significant variation was observed among the parental (chimeral) segregants, significant variation was observed among the dwarf (adventitious) somaclones (McPheeters and Skirvin, 1989). Somaclonal variation was noted for growth habit (dwarf to vigorous), flower

number per cluster (two to 200), fertility (0 to 83% drupelet set) and fruit quality. One of the pure thornless somaclones was patented and named 'Everthornless' in recognition of the fact that it was identical to its 'Evergreen' parent except that it did not have thorns (McPheeters and Skirvin, 1989, 1995, 2000; Norton, 1994; Norton and Skirvin, 1997). The fruit quality of 'Everthornless' is similar to that of 'Thornless Evergreen', but is less acidic and has more soluble solids than its predecessor (McPheeters and Skirvin, 1995, 2000).

To determine the stability of 'Thornless Evergreen' somaclones over time, to examine other traits associated with the thornless versus the thorny phenotypes and to establish baseline controls for later evaluations of regenerants from these plants, Norton and Skirvin (1997) analysed a sample of 7-year-old 'Thornless Evergreen' regenerants. They evaluated primocanes, leaves and flowers of plants from a selection of these *in vitro*-derived chimeral, intermediate and dwarf 'Evergreen' plants and compared them to thorny and thornless control parental plants. The intermediate and dwarf somaclones maintained their distinctive growth habits over 7 years in the field (Skirvin *et al.*, 1994), indicating that their growth habits were stable and not a transient effect of *in vitro* culture conditions (Norton and Skirvin, 1997). Although the thornless somaclones were stable for this trait, the degree and type of prickly-like structures varied considerably, indicating that the thornless gene (S_{te}) does not entirely suppress the production of prickles, but apparently alters their development. Increasing suppression was directly related to increasing dwarfism, suggesting a link between thornlessness and internode length.

Palonen *et al.* (2000) attempted to use somaclonal variation to isolate cold-hardy *Rubus* clones. They reported that cold-acclimatized raspberry clones behaved differently *in vitro* and *in vivo* (in small pots) and that they could detect differences in hardiness among cultivars *in vitro*. The results obtained in these studies did not relate to mature plants grown under field conditions.

5.2.2. Genetic transformation

The use of traditional breeding methods for *Rubus* is limited by a lack of suitable genes, the heterozygous nature of the various species and severe inbreeding depression (Mathews *et al.*, 1995). Recombinant DNA technology offers the potential to solve some breeding problems (Swartz and Stover, 1996).

Breeding objectives.

RBDV resistance. RBDV is the most serious virus disease of this crop species. Since RBDV is pollen-transmitted, its spread cannot be controlled by insect management strategies (Strik *et al.*, 2002). Resistance to RBDV is conferred by a single dominant gene (*Bu*), which has been widely exploited in breeding programmes; however, Barbara *et al.* (1984) identified a new virus strain that can overcome this resistance. Transformation with coat protein genes (and related strategies) may provide resistance to these diseases (Stelljes, 2000; Martin, 2002).

Resistance to grey mould. The grey mould (caused by *Botrytis cinerea*) is an important disease of *Rubus*. There is no good source of resistance to the disease. Control of grey mould is dependent upon periodical application of fungicides; however, the pathogen has begun to develop resistance to many of the most common fungicides. Engineered resistance is possibly the most sustainable method to address this problem.

Shelf-life. *Rubus* fruit have a very short shelf-life. The role of ethylene in bramble fruit ripening is unclear (Walsh *et al.*, 1983). Bramble fruits are reported to be non-climacteric, and do not respond to ethylene by ripening. On the other hand, Walsh *et al.* (1983) attempted to manipulate ethylene sensitivity and/or synthesis in *Rubus* to improve the shelf-life of the fruit. Alternative strategies, such as increasing the firmness of fruit, could extend shelf-life.

Protocol. *Agrobacterium tumefaciens*-mediated transformation has been used success-

fully with *Rubus*. Two different selectable marker genes have been used: hygromycin 3'-O-phosphotransferase (*hpt*), which confers resistance to hygromycin B, and neomycin 3'-O-phosphotransferase (*NPTII*), which confers resistance to kanamycin (Graham *et al.*, 1990; Hassan *et al.*, 1993; Mathews *et al.*, 1995; de Faria *et al.*, 1997; Kokko and Karenlampi, 1998). However, *Rubus* regenerants are very sensitive to kanamycin, compromising the regeneration of transformed plants. The use of geneticin7 instead of kanamycin in the *NPTII* transformation system and the use of hygromycin in the *hpt* transformation system appear to be promising solutions (Swartz and Stover, 1996).

The limitations of *de novo* regeneration and poor transformation rate of *Rubus* have prevented its extensive improvement by means of transformation (Hokanson, 2001). The recovery rate of transformed *Rubus* shoots is low and many recovered shoots are chimeral because shoots may have a multicellular origin. To obtain non-chimeral, transformed shoots, chimeral segregation methods must be used to break chimeras into homogeneous types that are more stable (McPheeters and Skirvin, 1983; Swartz and Stover, 1996).

Accomplishments. A few cases of successful transformation and transgenic plant recovery have been reported (Graham *et al.*, 1990; Hassan *et al.*, 1993; Mathews *et al.*, 1995; de Faria *et al.*, 1997; Kokko and Karenlampi, 1998; Meng *et al.*, 2001). Hassan *et al.* (1993) transformed a blackberry × raspberry hybrid with a gene for resistance to the contact herbicide chlorsulfuron.

Two strategies have been attempted to develop resistance to RBDV. Jones, A.T. *et al.* (1998), working with a resistance-breaking strain of RBDV, have transformed indicator plants with the *cp* gene in the sense and antisense orientations, as non-translatable RNA and as the putative polymerase gene. Enhanced, but incomplete resistance to infection was observed using this approach. Taylor and Martin (1999) have sequenced the *cp* gene, movement protein, three different mutations of the movement protein and non-translatable RNA from an S isolate (non-resistance-breaking). 'Meeker' red raspberry

was transformed with these genes in separate constructs, and Martin (2002) reported that the transgenic plants have remained virus-free after repeated grafting with RBDV-infected materials.

Swartz *et al.* (1993) and Swartz Stover (1996) attempted to incorporate the grape gene for stilbene synthase into *Rubus* to provide resistance to grey mould. Mathews *et al.* (1995) transformed raspberry with the enzyme polygalacturonase-inhibiting protein (PGIP) in order to increase firmness of fruit and lower the losses due to grey mould infection.

Mathews *et al.* (1995) incorporated the gene for S-adenosylmethionine hydrolase (SAMase) into 'Canby', 'Chilliwick' and 'Meeker' red raspberry. Although raspberry is non-climacteric, the rationale for this study is that removal of ethylene can delay fruit decay. Some of these plants have been established in soil and are currently undergoing field evaluations (Hokanson, 2001).

There is a real and perceived danger that genes from transgenic cultivars might escape into wild populations. Luby and McNicol (1995) explored this possibility by examining wild raspberry populations growing near (from 5 m to no more than 5 km) hybrid raspberries developed by conventional sexual methods 20 to 30 years earlier. They used two markers, a semidominant gene (L_1) affecting fruit size and plant morphology and the recessive gene s , which gives spinelessness. They found no evidence of the L_1 gene escaping; the s gene was detected at very low frequencies (estimated at 0.004) surrounding production areas; however, no evidence of escape was found in remote sites. They concluded 'that escape does occur following large-scale deployment but that gene flow events are probably infrequent and spread is localized for genes having probable neutral selective value'.

5.3. Cryopreservation

The extreme diversity and large numbers of species and cultivars associated with *Rubus* germplasm make it difficult to maintain col-

lections of these lines in either a greenhouse or the field. An alternative method would be to store bramble shoots and meristems for long periods of time *in vitro*. Reed and Lagerstedt (1987) and Reed (1988) reported methods to cryopreserve *Rubus* shoots. Chang and Reed (1999) reported studies on the long-term survival of *Rubus* plants after cryopreservation. Meristems are harvested from *in vitro* shoots and cold-acclimatized at -1°C for 1 week (Reed, 1990, 1993a,b). Shoot apices (0.8 mm) are excised for cryopreservation, held for 2 days at -1°C and then transferred to cryotubes containing 5% (v/v) dimethyl sulphoxide (DMSO) on ice. After 20 min, the cryovials are immersed into liquid nitrogen. For recovery, the cryovials are removed from liquid nitrogen and held in a water bath (45°C) for 1 min, followed by 2 min at 22°C . Meristems are rinsed in liquid medium containing 1.2 M sucrose and transferred on to plant growth medium for recovery (<http://www.ars-grin.gov/ars/PacWest/Corvallis/ncgr/tc/scrp.plan.html>). Reed (1990, 1993a,b) also reported medium-term storage of 250 *Rubus* accessions on minimal medium at 4°C in plastic bags or glass jars.

6. Conclusions

The genus *Rubus* is taxonomically diverse and includes plants from many parts of the world. Traditionally, bramble geneticists obtain new plants by carefully selecting parents and hybridizing them to produce genetic diversity, which could be fixed either by apomixis or by another asexual propagation method, e.g. cuttings and micropagation. Conventional breeding for bramble improvement will benefit from the use of molecular marker-based systems. In addition, molecular markers should improve our understanding of relationships among cultivars; assist in the choice of diverse parents; and help us to distinguish apomicts from sexual seedlings in segregating populations. Genetic transformation of *Rubus* cultivars using *Agrobacterium* has been accomplished; however, the low regeneration frequency and poor transformation rate of *Rubus* have

prevented its extensive use. Somaclonal variation, although random and undirected, has been demonstrated to have potential for *Rubus* improvement. Because it is possible to

use both sexual and asexual methods to improve *Rubus*, both systems should be useful to future geneticists who plan to improve this important fruit crop.

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19

Rutaceae

The *Rutaceae* family consists of trees, shrubs (mostly) and herbs whose distinguishing feature is the presence of glands containing aromatic oils, which may be found on leaves, stems, flowers and/or fruit (Watson and Dallwitz, 1992 onwards). Spines and winged petioles are also common. There are approximately 150 genera and 900 or more species in the family. *Rutaceae* species are found mostly

in tropical and subtropical regions, with the greatest diversity in Australia and South Africa. Fruit may occur as a capsule, drupe, samara, schizocarp, follicle or hesperidium (as in edible citrus). The economic importance of plants in the family lies in their edible fruit, particularly in *Citrus*, their essential oils and, less commonly, their ornamental value.

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19.1 Citrus Grapefruit, Lemon, Lime, Orange, etc.

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1. Introduction

1.1. Botany and history

There are many plant and fruit biotypes in the genus *Citrus* and related genera of the subfamily *Aurantioideae*. *Citrus* taxonomy is controversial (see below); the species designations as proposed by Swingle (1943) have been used in this discussion. Commercially important scion species include the sweet orange (*C. sinensis* (L.) Osb), the mandarin/tangerine/satsuma group (*C. reticulata* Blanco), the grapefruit (*Citrus* × *paradisi* Macf.), lemons (*C. limon* (L.) Burm. f.), and limes (*C. aurantifolia* Christm.). The citron (*C. medica* L.) and the pummelo (*C. grandis* (L.) Osb.) are *Citrus* species grown to a lesser extent but important locally and historically. The fruit of all of these citrus types varies widely in size, shape, colour and flavour. There are also other citrus types grown primarily as rootstocks, and discussed below. Clearly, then, there is plentiful genetic diversity within this genus and its relatives.

Citrus is an ancient crop, with records of human cultivation extending back to at least 2100 BC (Webber *et al.*, 1967; Scora, 1988). Therefore, it has been difficult for citrus taxonomists and historians to ascertain centres of origin and diversity due to natural and perhaps human-aided hybridization, wide

dispersion and the paucity of remaining wild citrus stands. The centre of origin and diversity of *Citrus* and its related genera is considered to be South-east Asia, especially eastern India, northern Myanmar and south-western China, but possibly ranging from north-eastern India eastward through the Malay archipelago, north into China and Japan and south to Australia (Tanaka, 1954; Webber *et al.*, 1967; Scora, 1975, 1988; Gmitter and Hu, 1990; Soost and Roose, 1996). The wild relatives of citrus are native to southeastern Asia, the East Indian Archipelago, New Guinea, Melanesia, New Caledonia and Australia.

The exact paths of dispersion of citrus from its regions of origin were also unknown until relatively modern times; however, citrus is mentioned in the ancient writings of many cultures (Webber *et al.*, 1967; Scora, 1975). It is believed that some citrus types, including citrons, sour oranges and lemons, were spread slowly (from AD 500 to 1300) through wide areas, including into Europe, by successive waves of invaders and travellers, e.g. Muslim armies, Arab traders, Crusaders and others moving along trade routes.

Although there was some amount of citrus grown in Europe during these centuries, the types available were probably bitter and used mostly as condiments. The Portuguese introduced one or more superior types of

sweet orange into Europe, probably in the 16th century. Although mandarins had been cultivated in China and Japan from ancient times, mandarin cultivars were first brought into England in 1805 by Sir Abraham Hume and they subsequently spread to the Mediterranean region (Scora, 1975; Soost and Roose, 1996).

Citrus is very sensitive to freezing temperatures; this limits its growth range, and unprotected trees could not survive in many parts of Europe. Therefore orangeries and other devices for citriculture were developed. European sailors also became aware that citrus consumption prevented scurvy during their voyages, and carried citrus fruit for consumption and seeds and trees to plant along trade routes. Columbus and Ponce de Leon and others carried various citrus fruits to the New World in the late 1400s and early 1500s. Citrus culture proliferated in Florida in the late 1700s, when the first commercial shipments were made. At about the same time, citrus was introduced into California, although it was much later that commercial production began there (Webber *et al.*, 1967). Thus, all commercially significant *Citrus* fruit types were first cultivated in the Old World, except for grapefruit, which is believed to have originated as a hybrid of pummelo and sweet orange in Barbados (Gmitter, 1995).

1.2. Importance

Citrus species are the most widely grown fruit in the world; world production in 2000 was estimated to be 63.8 Mt, more than bananas/plantains (*Musa*) (102.3 Mt), grapes (*Vitis*) (60.9 Mt) or apples (*Malus*) (58.0 Mt) (FAOSTAT, 2004). Currently, citrus is grown throughout tropical and subtropical regions of the world where there is enough water for growth and winter temperatures are moderate enough for tree survival.

1.3. Breeding and genetics

1.3.1. Taxonomy and genetic diversity

Swingle and Reece (1967) divided the orange subfamily *Aurantioideae* into two tribes: (i)

Clauseneae, very remote and remote citroid fruit trees; and (ii) *Citreae*, citrus and citroid fruit trees. In *Clauseneae*, only the species *Clausena lansium* (Lour.) Skeels, the Chinese wampee, is cultivated for its edible fruit. Intertribal hybridization and grafting are generally impossible. *Citreae* is divided into three subtribes; *Citrus* is placed in the subtribe *Citrinae*. Intersubtribal grafting and hybridization is also not generally successful (Swingle and Reece, 1967). The subtribe *Citrinae* is divided into three groups: group A, primitive citrus trees; group B, near-citrus trees; and group C, true citrus fruit trees. The horticultural characteristics of many of the genera in groups A and B have been poorly characterized, but some genera that have attractive characteristics for transfer to *Citrus* have been identified. For example, *Severinia* species are tolerant of salt (Cooper, 1961; Swingle and Reece, 1967), excess boron (Cooper, 1961), citrus nematode (Hutchison and O'Bannon, 1972) and *Phytophthora* (Hutchison and Grimm, 1973), all useful attributes in citrus rootstocks. These species are graft-compatible with citrus, but horticultural performance is poor (Wutscher and Schull, 1975; Wutscher and Dube, 1977). Within the *Citropsis* (group B) are species that show immunity to *Phytophthora*, tolerance of the burrowing nematode, xerophytic characteristics and graft-compatibility with citrus (Ford and Feder, 1960; Swingle and Reece, 1967).

Group C, the true citrus fruit trees, consists of six genera: *Fortunella* (kumquats), *Eremocitrus*, *Poncirus*, *Clymenia*, *Microcitrus* and *Citrus*. All of these genera, with the possible exception of *Clymenia*, are interfertile with *Citrus*. Together they represent a diverse and at least somewhat accessible gene pool for citrus improvement. Characteristics such as tolerance of cold, salt and drought, resistance to citrus and burrowing nematodes, *Phytophthora* and citrus tristeza virus (CTV), rest dormancy and short juvenile and fruit development periods are present among some of the members of the group C genera (Swingle and Reece, 1967; Soost and Roose, 1996; Bowman *et al.*, 2001).

A critical factor that makes taxonomic classification difficult within *Citrus* is its

Table 19.1.1. *Citrus* types designated by Swingle (1943) as species. Eight of the ten are cultivated.

Swingle taxonomy	Common name	Examples of cultivars
<i>C. aurantifolia</i> Christm.	Lime	Mexican, Key West Indian, Key, Bearss
<i>C. aurantium</i> L.	Sour orange	Chinotto, Seville
<i>C. indica</i> Tan.	A non-cultivated Indian species	
<i>C. maxima</i> Merrill; formerly <i>C. grandis</i> Osbeck	Pummelo, shaddock	Acidless, Thong Dee
<i>C. limon</i> (L.) Burm. f.	Lemon	Femminello, Lisbon
<i>C. medica</i> L.	Citron	Etrog, Corsican
<i>C. paradisi</i> Macf.	Grapefruit	Marsh, Redblush, Flame
<i>C. reticulata</i> Blanco	Mandarin, tangerine	Clementine, Dancy, Satsuma
<i>C. sinensis</i> Osbeck	Sweet orange	Valencia, Washington navel, Shamouti
<i>C. tachibana</i> (Mak.) Tan.	A non-cultivated Japanese species	

reproductive biology. Besides partial or complete pollen and/or ovule sterility and gametophytic self- and cross-incompatibility within the genus, many citrus types reproduce asexually by seed via nucellar embryony (Soost and Roose, 1996). The nucellus, a temporary nutritive tissue in developing seeds, gives rise to adventive embryos that are genetically identical to the maternal parent. Many nucellar embryos may be present, although usually only two or three develop to maturity; citrus types that display this trait are referred to as polyembryonic. Very frequently, the nucellar embryos outcompete the single zygotic embryo for space or nutrients in the ovule, so that no zygotic embryos are produced.

This aspect of citrus reproduction has greatly complicated taxonomic analyses in the genus. On the one hand, *Citrus* contains an enormous degree of variation, with abundant natural hybridization giving rise to a wide range of phenotypes, suggesting one species with many subspecies. On the other hand, because this type of facultative apomixis is so widespread in the genus, exchange of genes is often prevented, leading to reproductive isolation, a requirement for species designation. Further, there is an extremely high rate of bud and limb mutations ('sports') in the genus. Beneficial ones may be vegetatively propagated, and may in fact be ancient in origin. Thus, the biological concept of speciation where there is exchange of genes between members of a

species and barriers to such an exchange between species is difficult to apply to the *Citrus* genus (Vardi and Spiegel-Roy, 1978).

Until the mid-1970s, citrus taxonomists based their conclusions solely on morphological and geographical data. This led to major disagreements on the classification of species within *Citrus*. One definition of a species is that when populations of two kinds occur together without interbreeding, they are considered different species. Thus, some 'lumpers' believed that all citrus types belong to one large species since they are all graft-compatible and, were it not for the reproductive barriers described above, they are all interfertile. Swingle (1943) devised a system where ten species in the subgenus *Citrus* were recognized (Table 19.1.1). On the other hand, Tanaka (1954) defined 147 different species. A major difference in these two systems involved the treatment of mandarins; Swingle placed all mandarins except *C. tachibana*, a wild species of Japan, and *C. indica*, a wild species of India, in *C. reticulata*, while Tanaka separated mandarins into 36 species. The wide difference in number of species recognized in these two systems and some intermediate ones reflected opposing theories about what degree of morphological difference justified species status and whether presumed hybrids among naturally occurring forms should be given species status.

Modern techniques have been instrumental in deciphering the taxonomic situation in

Citrus. In the mid-1970s, a comprehensive phylogenetic study that evaluated 146 morphological and biochemical tree, leaf, flower and fruit characteristics was performed (Barrett and Rhodes, 1976). This study, and another by Scora (1975), suggested that only three citrus types, citron (*C. medica*), mandarin (*C. reticulata*) and pummelo (*C. grandis*; now *C. maxima*) constituted valid species. How then did the other citrus types arise and maintain their integrity and how closely are they related? Biochemical and molecular markers have provided much evidence. More than 20 isozyme loci have been genetically characterized (Torres *et al.*, 1978, 1982, 1985; Harai *et al.*, 1986a; Harai and Kajiura, 1987; Roose and Traugh, 1988; Xiang and Roose, 1988; Durham *et al.*, 1992; Asins *et al.*, 1995; Herrero *et al.*, 1996a,b). These markers have been very useful because in many cases the loci are highly polymorphic; e.g. Torres *et al.* (1985) characterized 12 isozyme loci that had a total of 39 alleles in citrus and related genera. Isozymes were later augmented with DNA markers, including restriction fragment length polymorphisms (RFLPs) (Durham *et al.*, 1992; Jarrell *et al.*, 1992; Federici *et al.*, 1998), random amplified polymorphic DNAs (RAPDs) (Cai *et al.*, 1994; Coletta-Filho *et al.*, 1998; Federici *et al.*, 1998; Nicolosi *et al.*, 2000), sequence-characterized amplified regions (SCARs) (Nicolosi *et al.*, 2000), inter-simple sequence repeats (ISSRs) (Fang *et al.*, 1997b; Sankar and Moore, 2001), and simple sequence repeats (SSRs) or microsatellites (Luro *et al.*, 1995; Kijas *et al.*, 1997). These markers have elucidated the taxonomic situation in *Citrus* as follows.

The limes (*C. aurantifolia* Christm.) include both acid and sweet cultivars (Swingle and Reece, 1967). In addition, there are two kinds of acid limes, the small-fruited Mexican (West Indian, Key) type and the large-fruited Tahiti (Persian) lime, which is triploid and therefore seedless. Limes are citrus biotypes that are believed to be apomictically perpetuated (Barrett and Rhodes, 1976). There are very few cultivars, and these are fairly homogenous; seven cultivars had invariant isozyme genotypes at four loci (Torres *et al.*, 1978). However, limes are also relatively heterozygous, e.g. they have

heterozygous genotypes at seven of the ten isozyme loci evaluated (Torres *et al.*, 1978, 1982). Barrett and Rhodes (1976) suggested that the lime probably arose from a trihybrid intergeneric cross involving *C. medica* (citron), *C. grandis* (pummelo) and a *Microcitrus* species. Nicolosi *et al.* (2000) found that all RAPD and SCAR markers present in Mexican lime were also present in citron or in *C. micrantha* (a small-flowered papeda, a citrus relative that produces inedible fruit), suggesting that these two species were the parents. The role of citron as a parent of limes is supported by RFLP data (Federici *et al.*, 1998).

There are three types of fruit grouped within the highly apomictic sour oranges (*C. aurantium* L.): the common type, which is used principally as a rootstock for sweet oranges and other citrus biotypes and for the preparation of marmalade (the well-known Seville oranges); the bittersweet orange, whose fruit is similar to the common type, but less acidic; and the variant bitter oranges, which are grown primarily as ornamentals and for their flowers, from which neroli oil is extracted (Swingle and Reece, 1967). Barrett and Rhodes (1976) found intraspecific affinity (correlation coefficients of traits measured) to be quite high within common sour orange, although the biotype appeared to be highly heterozygous. Also, of 15 accessions examined, two had a variant isozyme genotype; the others were identical (Torres *et al.*, 1978). This condition is maintained by facultative apomixis, and therefore sour orange is also not a true species. Barrett and Rhodes (1976) believed sour oranges to be of predominantly *C. reticulata* genotype introgressed with genes from *C. grandis*. This is supported by SCAR and RAPD analyses (Nicolosi *et al.*, 2000). Chloroplast DNA analyses (Southern blots probed with labelled tobacco chloroplast DNA or polymerase chain reaction (PCR) amplification of chloroplast sequences using universal primers) revealed that *C. aurantium*, *C. limon*, *C. paradisi*, *C. sinensis* and *C. grandis* had the same Southern hybridization pattern, while *C. reticulata* and *C. medica* each had a unique pattern (Green *et al.*, 1986; Nicolosi *et al.*, 2000). Pummelo appeared to be the maternal

parent, which is not unexpected since it is monoembryonic and produces zygotic seedlings (Nicolosi *et al.*, 2000).

The pummelo (*C. grandis* (L.) Osbeck; *C. maxima* Merrill.) is used as a dessert fruit in South-east Asia, but is mostly grown as a curiosity elsewhere (Swingle and Reece, 1967). This largest fruit of the *Citrus* genus may be pigmented or non-pigmented and acid or acidless. Pummelos are one of the three citrus types that Barrett and Rhodes (1976) proposed as a true species because pummelos are monoembryonic and yet have a well-developed discontinuity of traits from other citrus types, i.e. reproductive isolation. Although pummelo is primarily cross-pollinating, some clones produce relatively vigorous selfed progeny. *C. grandis* can be separated from other citrus types by its unique ISSR banding patterns (Fang *et al.*, 1998b). Barrett and Rhodes (1976) found intraspecific affinity to be quite high in this citrus type, and this, along with the propensity for self-fertilization, was believed to result because the species was relatively homozygous. Isozyme data support this because pummelo is homozygous at all ten loci examined; of 14 accessions, only three have variant isozyme patterns (Torres *et al.*, 1978). However, four random *C. grandis* cultivars screened by Deng *et al.* (1997) with a SCAR marker, SCADO8, were all found to be unique at this locus, so the species may possess greater diversity than previously concluded from isozyme studies alone. Inheritance studies of this specific locus were not conducted, so absolute conclusions regarding heterozygosity cannot be drawn. However, it is worth noting that the patterns of polymorphic fragments visualized on agarose gels were suggestive of multiple allelism, by virtue of insertions/deletions and/or point mutations.

The lemons (*C. limon* (L.) Burm. f.) consist of the common, acid varieties and a few sweet or acidless types (Swingle and Reece, 1967). Lemon was used medicinally in ancient times; today it is used mostly for juice and flavouring. Barrett and Rhodes (1976) found intraspecific affinity to be quite high in this species. They speculated that this biotype consists of a unique, original

apomictic type, with only minor mutational variations. Isozyme data support this; of 16 accessions, only two had a variant isozyme genotype (Torres *et al.*, 1978), and a later study also found little variation (Gulsen and Roose, 2001b). Barrett and Rhodes (1976) speculated that lemon is a complex hybrid similar to lime but carrying a greater proportion of citron genes. Molecular marker data indicate that lemon originated from citron and sour orange, with sour orange being the maternal parent (Nicolosi *et al.*, 2000; Gulsen and Roose, 2001a). When ISSR markers were examined, a total of 12 polymorphic fragments generated by seven primers were detected among six lemon cultivars, suggesting a possible polyphyletic origin for lemon (Fang *et al.*, 1998b; Gulsen and Roose, 2001b). Six of ten isozyme loci examined were heterozygous (Torres *et al.*, 1978, 1982).

The citrons (*C. medica* L.) also fall into acid and sweet classes, with several varieties in each class (Swingle and Reece, 1967). Citrons are grown mostly for their peel, which is candied. The fruit has had religious significance for some cultures. Citron is the second type that Barrett and Rhodes (1976) advanced as a true species. It is monoembryonic. It is also relatively homozygous; eight of ten isozyme loci are homozygous and, of six cultivars, one is variant (Torres *et al.*, 1978, 1982). DNA marker data support species status (Nicolosi *et al.*, 2000). Citron has a unique chloroplast hybridization pattern (Green *et al.*, 1986; Nicolosi *et al.*, 2000), also supporting species status. Interestingly, the chloroplast data of Nicolosi *et al.* (2000) indicated that citron always acted as the male parent, which is unexpected given the monoembryonic nature of this species.

Grapefruit (*Citrus* $\times$ *paradisi* (*C. paradisi* Macf.)) is the only citrus biotype in which a hybrid origin and subsequent selection for mutants are well documented. RAPD and SCAR marker data indicate that grapefruit was derived from a back-cross between sweet orange and pummelo, as do historical and morphological data (Bowman and Gmitter, 1990a,b; Gmitter, 1995; Nicolosi *et al.*, 2000). Not unexpectedly, intraspecific affinity is very high in this biotype (Barrett and Rhodes, 1976). When isozymes have

been used to examine 13 cultivars, no variation has been detected (Torres *et al.*, 1978); when 1230 ISSR markers have been used to characterize seven cultivars, one was different from the norm, which was attributed to mutation (Fang and Roose, 1997). Surprisingly, grapefruit may be rather homozygous; eight of ten isozyme loci were homozygous in this biotype (Torres *et al.*, 1978).

Mandarins (*C. reticulata* Blanco) are the most phenotypically heterogeneous group in citrus; both monoembryonic and polyembryonic clones exist, as do self-fertile and self-incompatible types (Swingle and Reece, 1967). This suggested to Barrett and Rhodes (1976) that a broader-based, more complex heterozygosity was present than exists in the other facultatively apomictic types. Therefore, this was the third citrus type assigned species status. Sweet mandarin types have been used for dessert fruit since ancient times, while sour types have been used as rootstocks and for flavourings and medicine. Thus, it is difficult to assess the relative importance of genetic versus mutational variation in the complex history of this species. SCAR and RAPD analyses did not allow the identification of a progenitor type (Nicolosi *et al.*, 2000). Although the molecular marker data reveal great heterogeneity within this group, they support species status (Torres *et al.*, 1978; Luro *et al.*, 1995; Coletta-Filho *et al.*, 1998; Fang *et al.*, 1998b; Nicolosi *et al.*, 2000). For example, when RAPD markers were used to evaluate genetic similarity among 35 mandarin accessions, the minimum Jaccard coefficient was 0.77, which indicates a high genetic similarity in this group. The researchers proposed that the mandarin group is a single species, composed of several genetically different individuals and a great number of hybrids (Coletta-Filho *et al.*, 1998). Finally, *C. reticulata* has a unique chloroplast banding pattern, again suggesting that it should have species status (Green *et al.*, 1986).

Sweet orange (*C. sinensis* Osbeck), the most widely grown and consumed citrus type, presents something of a mystery. Four kinds of sweet oranges are recognized: the common round, or blond, orange, which is the most important and of which there are

many varieties; the acidless orange, of minor importance; the blood orange, which has a red pigmentation in the flesh due to the accumulation of anthocyanins; and the navel orange, grown for fresh consumption (Swingle and Reece, 1967). They can also be categorized on the basis of season of maturity as early, mid-season or late. Barrett and Rhodes (1976) observed much lower intraspecific affinity in this type than in any of the other types they considered facultative apomicts (sour orange, lemon and grapefruit); in other words, there was a relatively great diversity in phenotype. Some studies indicate that, genetically, sweet oranges are a biotype. Chromosome banding patterns of ten clones were heterozygous and invariant (Pedrosa *et al.*, 2000). When three microsatellite probes were assayed, no differences were detected among ten cultivars (Luro *et al.*, 1995). More than 300 RAPD primers were used to examine five phenotypically distinct cultivars, but no consistent or repeatable differences were identified (F.G. Gmitter and S.Y. Xiao, unpublished data). This points to a monophyletic origin for sweet orange, followed by somatic mutation and selection of desirable clones. However, when four isozyme loci were used to examine 21 cultivars, there were seven variants at one locus (Torres *et al.*, 1978). Further, of 31 cultivars, 14 differed from the basic ISSR profile of about 1230 fragments by one to four ISSR bands (Fang and Roose, 1997). Fang and Roose (1997) believed these differences originated via mutation. However, it seems unusual that small mutations, which perturb only a small part of the genome, could be detected at such high frequencies using molecular markers. None the less, it also seems unlikely that more than 1200 presumably identical fragments could have been accumulated randomly and from polyphyletic sources in the diverse sweet orange cultivars examined. It seems more plausible that the lower level of sweet orange intraspecific affinity reported is the result of the passage of more time since the progenitor's origin (perhaps as many as 3000 years), compared with the origins of the grapefruit (< 300 years). From the breeding and cultivar improvement perspective, the 'biotype species', i.e. grapefruit, sweet

orange, sour orange, lemon and lime, are best considered to be unique interspecific hybrids derived by varying degrees of introgression among the true biological species, i.e. pummelo, citron, mandarin, etc. This limits, or excludes in some cases, the utilization of sexual hybridization as a means to genetic improvement, which in turn has profound implications for the potential benefits of biotechnology for cultivar improvement in these groups.

Like sour orange, sweet oranges are thought to be predominantly of *C. reticulata* (mandarin) genotype introgressed with *C. grandis* (Barrett and Rhodes, 1976; Nicolosi *et al.*, 2000). The sweet orange and sour orange biotypes are thought to have a parallel but separate origin, with their differences stemming from parentage from separate subspecies from within the polytypic *C. reticulata* or distinct individuals of *C. grandis*. Similar microsatellite patterns observed with sweet oranges and mandarins agree with the close phylogenetic relationships of these species (Luro *et al.*, 1995).

1.3.2. Rootstocks

Major breeding objectives. Candidate selections must be tolerant of various physiological and biological stresses associated with root systems. They should be graft-compatible with most commonly grown scion cultivars. They should produce fruit that are reasonably seedy, and the seeds should contain predominantly nucellar embryos, because citrus rootstocks are almost exclusively propagated from seeds. Most importantly, they must produce grafted trees capable of yields as great as those of rootstock currently used, with little or no decrease in relative fruit quality characteristics. Determination of the horticultural performance of rootstock candidates demands extensive replicated field trials in various environments. Currently there are few seedling procedures that can adequately predict field performance of mature trees for any characteristic.

Breeding accomplishments. *Citrus* is diploid ($2n = 2x = 18$), with a small genome

(about 385 Mb/haploid) (Arumuganathan and Earle, 1991), although some natural polyploids have been observed and others have been created for breeding purposes (see below). Except for the problems with reproductive biology described above, *Citrus* spp. and those of the group C closely related genera can be easily hybridized. However, reproductive barriers are great in some cases. Further, the long juvenile period of most *Citrus* seedlings is as great an impediment to evaluation and selection as nucellar embryony is to recombination. Even after flowering begins, there is a period of settling down during which tree and fruit characteristics become more stable and typical of mature tree performance. Subsequent advanced tests of scion/rootstock combinations are necessary for both potential scion and rootstock cultivars. The great heterozygosity of most *Citrus* clones results in substantial and unpredictable genetic segregation in hybrid progeny, when hybrids can be made. Inbreeding depression has been observed among progeny from various cross combinations and following selfing of certain self-fertile clones, as expected from such heterozygous, outcrossing plants (Soost and Roose, 1996).

Thus, clonal selection has been the major force in the historical development of *Citrus* cultivars. Very few of the currently grown cultivars have had their origin in planned breeding programmes; nearly all of the important rootstock and scion varieties originated as chance seedlings, or bud sport mutations in the case of some scion cultivars (Hodgson, 1967); however, hybridization has also played a part in citrus rootstock development. For example, citranges (*C. sinensis* × *Poncirus trifoliata*) and citrumelos (*Citrus* × *paradisi* × *P. trifoliata*) were created with the objective of transferring cold tolerance to citrus (Soost and Roose, 1996). Although cold-tolerant hybrids were obtained, these were not acceptable as scions because of the accompanying poor fruit quality. The hybrids were subsequently evaluated as rootstocks. 'Carrizo' and 'Troyer' citranges and 'Swingle' citrumelo rootstocks are now widely used because of their *Phytophthora*, virus, and nematode tolerance.

1.3.3. Scions

Major breeding objectives. Productive new selections that retain the primary characteristics associated with established cultivars but produce higher yields, earlier or later maturing fruit or improvements in fruit quality are sought. These fruit quality aspects are specific to cultivar groups and also are determined by utilization or marketing of fruit or fruit products. Sweet oranges that have better processing characteristics are desired by juice producers; decreases in limonin content or levels of pectin methyl esterase, or improved flavour profiles after pasteurization, can be important. Fresh market oranges could be improved by developing fruit with rinds that are more easily removed, better cosmetic appearance, seedlessness and better flavour and texture. Grapefruit consumption and marketing trends have moved towards more deeply pigmented fruit. Cultivars with improved early season quality, or those with the ability to retain high quality later into the on-tree storage season, could extend the market and increase consumer acceptance. For mandarin cultivars, the essential objectives of all the world's citrus breeding programmes are clearly seedlessness and easily removed rinds. Improvements in colour, flavour and texture and the absence of off-flavours following storage and shipping are also important. For all cultivars, the incorporation of resistance to a host of diseases and pests can be critical to the success of new cultivars, and in certain cases these traits may be vital to the survival of some citrus industries.

Breeding accomplishments. The probable hybrid nature of the economically important scion species *C. sinensis* and *Citrus* × *paradisii* has been discussed above. Sexual hybridization has been futile for sweet orange and grapefruit improvement. Likewise, no lemon or lime cultivars have been developed via hybridization and selection. A consequence of their hybrid origin is that development of genetically improved cultivars of these types cannot be accomplished except by mutation breeding or introgression of genes from other species, e.g. *C. reticulata*; however,

marketing and regulatory demands may preclude utilization of the latter approach. For these citrus types, alternatives to hybridization and selection are essential for genetic advancement. Clonal selection within cultivar groups, i.e. budline selection, has been practised extensively in many citrus growing areas and has been particularly useful for the development of improved strains of cultivar groups like satsuma mandarin and sweet orange. The derivation of all currently grown grapefruit cultivars can be traced through a series of seedling and budsport mutations (Bowman and Gmitter, 1990a,b; Gmitter, 1995).

Sexual hybridization has been most successful for mandarin cultivar development (Hodgson, 1967; Soost and Roose, 1996). Many *C. reticulata* hybrids, including inter-specific tangors (mandarin × sweet orange) and tangelos (mandarin × grapefruit) have been developed and released by breeding programmes. Sexual hybridization should continue to be useful in mandarin improvement because of increased numbers of monoembryonic breeding parents.

2. Molecular Genetics

2.1. Gene cloning

The genetic nature and mode of inheritance of most important *Citrus* traits are unknown. This is not surprising considering the aspects of reproductive biology discussed above. Making the appropriate crosses to determine genetic segregation of important traits is extremely difficult; however, in the last several years, with the molecular tools available, many genes have been cloned and characterized to some extent. Except for significant efforts to clone genes involved in disease resistance, described in the following section of this chapter, many of the genes that have been cloned have or are suspected to have some effect on fruit production or maturation. A number of genes have been cloned and examined for their effect on acid and sugar accumulation during fruit ripening. A single-copy mitochondrial citrate synthase gene has been identified by homology with

the gene from other species (Canel *et al.*, 1995, 1996), but the gene does not co-segregate with the acidless fruit phenotype of pummelo 2240 (Canel *et al.*, 1996). Further studies with other acidless and acid-accumulating fruits have suggested that changes in acid accumulation are not due to differences in the activity of citrate synthase (Sadka *et al.*, 2000b, 2001). Genes for aconitase (Sadka *et al.*, 2000a) and nicotinamide adenine dinucleotide phosphate (NADP⁺)-isocitrate dehydrogenase (Sadka *et al.*, 2000c) have also been cloned through homology with *Arabidopsis* and potato, respectively, and their activities in ripening fruit partially characterized. Three partial complementary DNAs (cDNAs) encoding sucrose phosphate synthase (SPS) have been isolated from satsuma mandarin fruit and leaves by reverse transcription (RT)-PCR using primers designed from sequences conserved in other plants (Komatsu *et al.*, 1996). Sequence data, restriction enzyme patterns, Southern analyses and Northern blots showed that, although the three cDNAs were all predicted to be SPS, they were clearly different forms and were differentially expressed in tissues, organs and developmental periods (Komatsu *et al.*, 1996, 1999). A vacuolar H⁺-adenosine triphosphatase (ATPase) 69 kDa catalytic subunit that may be involved in sugar transport into the vacuole and/or cell expansion has also been cloned and characterized (Takanokura *et al.*, 1998).

Genes that may affect fruit colour and flavour are also being isolated. Genes that function in the biosynthesis or regulation of carotenoids, which are the compounds responsible for colour production in *Citrus* fruit, are being identified and characterized (Moriguchi *et al.*, 1998a; Ikoma *et al.*, 2001; Kita *et al.*, 2001; G.A. Moore, unpublished data). Flavonoid biosynthesis is important in citrus fruit flavour, leading in some types to bitter tasting flavonones such as naringin. The genes that function in the early part of the pathway have been cloned and their expression is correlated with flavonoid accumulation (Moriguchi *et al.*, 1999, 2001; G.A. Moore, unpublished data); chalcone synthase has been found to consist of a small, differentially expressed gene family, while

chalcone isomerase appears to be encoded by a single gene (Moriguchi *et al.*, 1999). Another biochemical pathway that is responsible for bitterness in citrus fruit is that of limonoids, and a limonoid uridine diphosphate (UDP)-glucosyl transferase has been cloned (Kita *et al.*, 2000a). Its expression fluctuates with the amount of limonin glucoside in developing fruit.

Another area that has received a great deal of attention is stress, both biotic and abiotic. A cDNA associated with citrus blight encodes a protein of unknown function but with similarity to expansin (Ceccardi *et al.*, 1998). The gene was cloned by the laborious method of identifying a unique protein in blighted tissue, partially sequencing it, producing a partial DNA sequence by PCR with degenerate primers and screening of a cDNA library. A phospholipid hydroperoxide glutathione peroxidase (PHGPX) clone was isolated from a *C. sinensis* cDNA expression library prepared from mRNA isolated from a salt-treated cell suspension (Holland *et al.*, 1993a,b). This gene and its resulting protein are the most thoroughly characterized gene in *Citrus* thus far, and the most thoroughly examined PHGPX in plants (Ben-Hayyim *et al.*, 1993; Beeor-Tzahar *et al.*, 1995; Eshdat *et al.*, 1997; Gueta-Dahan *et al.*, 1997; Avsian-Kretchmer *et al.*, 1999). Other cloned genes implicated in salt stress include an atypical Lea5 (Naot *et al.*, 1995b) and an oleosin homologue (Naot *et al.*, 1995a).

The response of *Citrus* to acclimatizing and freezing conditions is being studied (G.A. Moore, Gainesville, Florida, USA, unpublished data). Cold acclimatization-induced changes in freezing tolerance and translatable RNA content have been compared in seedlings of cold-sensitive *C. grandis* Osb. 'Thong Dee' (pummelo) and a very cold-hardy citrus relative *P. trifoliata* 'Pomeroy' (trifoliolate orange) (Durham *et al.*, 1991). Cold acclimatization of pummelo (10 days at 15°C followed by 4 weeks at 10°/5°C day/night) results in a decrease in LT₅₀ from -6 to -8°C. In trifoliolate orange acclimatized for 7 weeks at 5°C, the LT₅₀ decreases from -9 to -18°C. Qualitative changes of the *in vitro* translation profile, revealed by two-dimensional gel electrophoresis, have been

observed following cold acclimatization of both species. An mRNA for a large polypeptide (c. 160 kDa) was detected following cold acclimatization of trifoliolate orange. A similar change was not observed following cold acclimatization of pummelo. Six cDNAs representing unique cold-induced sequences have been cloned from a cDNA library constructed from cold-acclimatized 'Pomeroy' trifoliolate orange (Cai *et al.*, 1995). These cDNAs are differentially expressed in cold-acclimatized pummelo and trifoliolate orange. Two of the sequences, pBCOR115 and pBCOR119, belong to the same gene family with similarity to group 2 late embryogenesis abundant (LEA) proteins. Expression of the single gene characterized further was also up-regulated during salinization, but repressed during drought and flooding stress.

Chitinases are induced by a variety of chemical elicitors or pathogen infections and are believed to be involved in defence responses in plants. A cDNA of a class II acidic chitinase was isolated from a sweet orange callus cDNA library by probing with a tobacco homologue (Nairn *et al.*, 1997). Another pathogenesis-related gene, β -1,3-endoglucanase, has also been identified (Porat *et al.*, 2002). A cDNA homologue to the human *defender against apoptotic death* gene (*dad-1*) has been isolated from mandarin fruit using a heterologous probe from apple (Moriguchi *et al.*, 2000).

Genes that are involved in the synthesis or functions of hormones have been isolated and characterized under certain conditions. Two 1-aminocyclopropane-1-carboxylate (ACC) synthase genes, which are involved in the synthesis of ethylene, are expressed in sweet orange fruit peel; one is chilling-induced and one is chilling-repressed (Wong *et al.*, 1999). When a Carrizo citrange gibberellin 20-oxidase was ectopically expressed in transgenic tobacco, the plants were taller, had larger inflorescences and synthesized more gibberellin than control plants; the transgenic plants were unresponsive to exogenously applied gibberellic acid (GA₃) (Vidal *et al.*, 2001).

Genes other than the ones described above that are involved in fruit development

and physiology have been cloned and characterized. Changes in the levels of mRNA for the putative growth-related genes endoxyloglucan transferase-related protein, extensin, β -1,3-glucanase, glycine-rich protein, pectinacetyltransferase and pectin esterase isolated from a rapid fruit development stage cDNA library have been characterized (Kita *et al.*, 2000b). Gene expression in abscission zones is being examined, and cellulase and pectin methyltransferase genes from sweet orange have been cloned following partial amino acid sequencing of the relevant proteins (Burns *et al.*, 1998; Nairn *et al.*, 1998).

Genes that do not fit into any specific category have been cloned from various citrus types. For example, two genes of unknown function that display differing diurnal expression and light response have been identified in 'Duncan' grapefruit (Abied *et al.*, 1994). A non-photosynthetic ferredoxin small gene family, of which at least one member is induced by ethylene, has been characterized (Alonso *et al.*, 1996); a putative vacuolar processing thiolprotease, which is also induced by ethylene, was additionally cloned (Alonso and Granell, 1995). When differential display was applied to samples extracted from ethylene-treated and non-treated fruit peel, a gene that may be involved in the biosynthesis of thiamine was identified (Jacob-Wilk *et al.*, 1997). A chlorophyllase, the first enzyme in the chlorophyll biosynthetic pathway, has been cloned from sweet orange following partial purification and sequencing of the protein (Jacob-Wilk *et al.*, 1999). An intensive molecular characterization of seed formation and polyembryony *in vivo* and *in vitro* is under way; it is hoped that this will result in the production of less seedy fruit (Koltunow *et al.*, 1996, 1997; Koltunow and Brennan, 1998).

In summary, a number of genes from various citrus types have been cloned and characterized to some extent. Many of the genes described above were obtained using relatively laborious techniques, e.g. protein purification and sequencing prior to cloning of cDNAs. In other cases, knowledge of gene sequences from other species was essential for the isolation of citrus sequences. As functional genomic methods are developed for

citrus, a wider repertoire of genes will become available (see below).

2.2. Mapping, marker-assisted selection and positional cloning

Molecular markers have been used to elucidate citrus phylogeny (see above). Molecular markers have also been employed to distinguish individual cultivars (Elisiario *et al.*, 1999a,b; Protapadakis and Papanikolaou, 1999; Rahman *et al.*, 2001). They can be used to distinguish zygotic from nucellar types in populations of rootstock seedlings (Harai *et al.*, 1986b; Ashari *et al.*, 1988; Khan and Roose, 1988; Moore and Castle, 1988; Xiang and Roose, 1988), in budded trees in established groves or rootstock trials (Roose and Traugh, 1988; Anderson *et al.*, 1991) and following hybridization with polyembryonic maternal parents (Carimi *et al.*, 1998a; Ruiz *et al.*, 2000). Molecular markers can also verify genotypes following somatic hybridization (Grosser and Gmitter, 1990). RAPD markers have been used to analyse citrus chimeras (Sugawara *et al.*, 1995). Molecular markers have also been useful for elucidating the fact that the dwarfing phenotype of 'Flying Dragon' *P. trifoliata* rootstock is probably due to a single dominant gene (Cheng and Roose, 1995) and for surveying the genetic diversity in this species (Fang *et al.*, 1997b).

Molecular markers have been used to characterize the citrus genome (Liou *et al.*, 1996). Restriction enzyme digestion followed by RAPD amplification allowed the analysis of cytosine methylation in citrus (Cai *et al.*, 1996). It was possible to detect 28 methylation events involving 23 amplified bands with seven random primers and two pairs of enzymes, and one locus influencing cytosine methylation was identified and mapped. Copia-like retrotransposon sequences have been detected in the citrus genome (Asins *et al.*, 1999), as have specific repetitive sequences (Matsuyama *et al.*, 1996, 1999, 2001; Fann *et al.*, 2001).

Several linkage maps have been developed in citrus to improve genetic analysis of various traits, facilitate marker-aided selection (MAS) and allow positional cloning of

important genes (Durham *et al.*, 1992; Jarrell *et al.*, 1992; Cai *et al.*, 1994; Luro *et al.*, 1995; Kijas *et al.*, 1997; Cristofani *et al.*, 1999; Fang and Roose, 1999; Garcia *et al.*, 1999; M. Roose, University of California Riverside (UCR), personal communication). However, most maps are low density, with only the University of Florida (UF) (Cai *et al.*, 1994; F.G. Gmitter, Jr, UF Citrus Research and Education Center (CREC), personal communication) and UCR (M. Roose, UCR, personal communication) maps having > 100 markers. New maps using amplified fragment length polymorphism (AFLP) and ISSR markers are being developed that contain several hundred markers (F.G. Gmitter and G.A. Moore, UF, unpublished data). All mapping populations have been relatively small (< 60 progeny), and, despite efforts to share RFLP probes and map with common RAPD primer sets, the UF and UCR maps share only 30 markers (M. Roose, UCR, personal communication). Development of improved maps with more shared markers is therefore an important objective for citrus geneticists.

The value of genetic linkage maps for citrus genetic studies and for use in genetic improvement schemes has been enhanced by mapping several genes and quantitative trait loci (QTLs) for traits of economic importance. A localized map of the region surrounding a gene, *Ctv*, which confers resistance to CTV, was developed by Gmitter *et al.* (1996), and has been useful for MAS. Other important traits have been targeted, and single genetic loci have been mapped for *acitric* (a gene conferring lower juice acidity) (Fang *et al.*, 1997a) and rootstock-mediated scion dwarfing (Cheng and Roose, 1995). Cold-acclimatization-responsive QTLs have been mapped in the base map population first used by Durham *et al.* (1992) (Cai *et al.*, 1994; Weber, 1999). A major QTL for resistance to citrus nematode (*Tylenchulus semi-penetrans*), designated *Tyr1*, was identified by Ling *et al.* (2000). The back-cross population of pummelo × (pummelo × trifoliolate orange) was salinized and 73 potential QTLs with log of odds (LOD) scores higher than 3.0 that influenced the accumulation of Na and/or Cl in salinized or non-salinized plants were mapped, resulting in the identi-

fication of 17 regions of the citrus genome that may contain QTLs of large effect (Tozlu *et al.*, 1999b). A further large group of QTLs for morphological traits under the same conditions was placed on the map and many correlations between the two groups were identified (Tozlu *et al.*, 1999a). Additional mapping is under way at the UF and several QTLs have been identified with important effects on morphology, plant vigour and cold tolerance (Sahin-Cevik, 1999; Weber, 1999). QTL studies have also been done to investigate the genetic control of apomixis (Garcia *et al.*, 1999) and of yield and seed number (Garcia *et al.*, 2000).

Much emphasis has been placed on cloning the dominant gene *Ctv*, which provides resistance to CTV, because of the threat this virus poses to citrus industries. This gene is present in the heterozygous form in *P. trifoliata* (a sexually compatible citrus relative). Although this gene can be transmitted by hybridization to *Citrus*, introgression into commercially acceptable scion cultivars is a daunting challenge for the reasons described above. Once cloned, *Ctv* could be transferred to the genomes of susceptible citrus cultivars using available genetic transformation technology (see below). Since the product of this gene is unknown, a positional (or map-based) cloning strategy has been adopted in several laboratories, principally those of F.G. Gmitter, Jr (UF CREC) and M.L. Roose and T.E. Mirkov (UCR and Texas A&M). The first step has been to identify molecular markers tightly linked to *Ctv*; this has been accomplished by generating high-resolution linkage maps of the region surrounding the gene, using molecular markers and segregating progeny populations (Gmitter *et al.*, 1996; Mestre *et al.*, 1997a; Fang *et al.*, 1998a). The mainly dominant markers were converted into more reliable co-dominant markers, which delimited a small region around *Ctv* (Deng *et al.*, 1997; Fang *et al.*, 1998a). Besides their use in positional cloning these markers can be used in MAS.

However, small regions in cM still translate into large regions that have to be traversed when moving from marker to *Ctv* (Gmitter *et al.*, 1998). Therefore, large insert bacterial artificial chromosome (BAC)

libraries have been developed (Deng *et al.*, 2001b; Yang *et al.*, 2001). One library was constructed from genomic DNA from a single intergeneric hybrid individual heterozygous for the dominant allele (Deng *et al.*, 2001b), and the other from an individual *Poncirus* seedling homozygous for the resistance allele (Yang *et al.*, 2001). One library consists of 45,696 clones with an average insert of 80 kb (Yang *et al.*, 2001), while the other library contains 24,576 clones with an average insert size of 115 kb (Deng *et al.*, 2001b), corresponding to 9.6 and 7 genome equivalents, respectively. The researchers then 'walked' from molecular marker loci towards *Ctv* by searching for clones that contained the marker loci and then arranging them in order by using BAC insert ends or other sequences as probes (Chen and Gmitter, 1999; Yang and Mirkov, 2000). This area of the genome has been arranged into contiguous sequences (contigs). In one case a contig of approximately 1.2 Mb was identified (Yang *et al.*, 2001); the other group developed a contig of 550 kb in length (Deng *et al.*, 2001b). These distances were further narrowed down to regions of 300 and 180 kb, respectively (Deng *et al.*, 2001b; Yang *et al.*, 2001). These regions are still large enough to contain many open reading frames, and indeed sequence analyses have revealed several putative R gene sequences (Z. Deng and F.G. Gmitter, Jr, unpublished data; T.E. Mirkov and M.L. Roose, personal communication). Cosmid or other large insert vectors are being used to introduce sequences from this region into susceptible cultivars to identify which of these sequences is the authentic *Ctv* (F.G. Gmitter, Jr, UF CREC, unpublished data). Positional cloning efforts are also being pursued to clone *acitric* (M.L. Roose, UCR, personal communication).

The *Ctv* region contains multiple loci with similarity to disease resistance genes previously identified in other species. Additionally, it has been found that the citrus nematode resistance QTL, *Tyr1*, is closely linked to *Ctv* (Ling *et al.*, 2000). Many resistance gene candidate (RGC) sequences are associated with known genes conferring resistance to viruses, bacteria, fungi or nematodes, making them very useful in mapping

and cloning resistance genes (Speulman *et al.*, 1998). A PCR approach with degenerate primers designed from the nucleotide-binding site (NBS) motifs of disease resistance genes has been used to clone homologous *Citrus* genes, an approach that has resulted in the cloning of several hundred RGC sequences in other plant species (Bent *et al.*, 1994; Aarts *et al.*, 1998; Shen *et al.*, 1998; Deng *et al.*, 2000). Deng *et al.* (2000, 2001a,b) cloned 24 RGC sequences (grouped into 13 classes) from an intergeneric hybrid of *Poncirus* and *Citrus* and found that they share strong similarities with the NBS-leucine-rich repeat (LRR) class, kinase class or LRR class disease resistance genes in GenBank. One RGC co-segregates with *Ctv* and another is tightly linked to the major locus, *Tyr1*, which controls citrus nematode resistance (Ling *et al.*, 2000; Deng *et al.*, 2001b). Using cloned RGCs as probes to screen their BAC libraries, this group identified nearly 500 BAC clones containing RGC sequences (Deng *et al.*, 2001b; F.G. Gmitter, Jr, UF CREC, personal communication). Preliminary data suggest that some of these sequences are actual resistance genes. The M.L. Roose/T.E. Mirkov group has identified some RGCs during the course of BAC sequencing and *Ctv* contig development. More recently, RGCs with substantial similarity to and identity with *Xa21*, a kinase class gene for resistance to a *Xanthomonas* pathogen of rice, have been cloned (F.G. Gmitter, Jr, unpublished data). Citrus canker, which is a serious disease in many of the citrus production areas of the world, is also caused by a species of *Xanthomonas*, and application of this tool for engineering canker resistance into scion cultivars may be considered.

2.3. Functional genomics

Several groups have been sequencing expressed sequence tags (ESTs). These are clones from cDNA libraries that should contain the partial or full-length coding regions of genes expressed in the tissue or developmental stage sampled or in tissue after exposure to various physiological or environmental conditions. These citrus sequences

are being entered into GenBank, but little has been published about them yet. The exception is a group in Japan that has been sequencing for some time and has published data on the kinds of gene repertoires they are identifying from individual cDNA libraries (Hisada *et al.*, 1997, 1999; Moriguchi *et al.*, 1998b). Of 950 clones sequenced from a library from rapidly developing fruit, 426 showed sequence homology with previously characterized genes from other species (Hisada *et al.*, 1997), while 195 of 297 total clones from a mature fruit cDNA library had a predicted function (Moriguchi *et al.*, 1998a). However, these analyses were done some time ago and the numbers of sequences with homology would probably be higher now. Many of the identified genes were 'house-keeping enzymes'.

3. Micropropagation

Citrus can be micropropagated via either organogenesis or somatic embryogenesis (see below). Some researchers have reported that rooting or grafting of shoots following regeneration and acclimatization can be problematic (Parthasarathy *et al.*, 1999).

4. Micrografting to Eliminate Viruses

Citrus trees are usually propagated by grafting a bud of a scion cultivar on to a seedling rootstock. This propagation method allows the transmission of virus and mycoplasma diseases, including exocortis, psorosis, tristeza, xyloporosis, greening and stubborn (Navarro *et al.*, 1975; Navarro, 1981). Since citrus viruses are rarely seed-transmitted, virus elimination from polyembryonic clones can be accomplished by selection of true-to-type nucellar seedlings. However, some citrus clones are monoembryonic, producing zygotic seedlings, and many commercially desirable cultivars are seedless.

The technique of meristem culture for the production of virus-free plants has been unsuccessful with citrus, although multiple shoot formation from apical cultures has been reported (Kitto and Young, 1981;

Barlass and Skene, 1982). A modified technique, *in vitro* shoot tip grafting, was developed by Murashige *et al.* (1972) and refined by Navarro *et al.* (1975). Aseptically grown, etiolated, 2-week-old nucellar seedlings are used as rootstocks. The seedlings are decapitated, their cotyledons are removed and the root is shortened. Shoot tips containing the apical meristem and three leaf primordia are isolated from new shoots of the desired scion. An inverted T-incision is made on the epicotyl of the rootstock seedling and the shoot tip is placed in the incision. Grafted plants are allowed to develop aseptically in a nutrient solution for several weeks before being transferred to soil. Grafted plants are largely virus-free and are not juvenile. This technique is now being used in citrus improvement programmes throughout the world to obtain virus-free budwood for commercial propagation. In addition, shoot tip grafting of imported bud material is also effective in reducing the risk of introducing diseases from one country to another (Navarro, 1981).

Several factors influence the rate of success of grafting or virus elimination. The frequency of successful grafts increases with the size of the shoot tip, but the percentage of virus-free plants declines, although this depends on the pathogen. Therefore, the shoot-tip size chosen reflects a compromise between these two criteria (Navarro *et al.*, 1975; Navarro, 1981). Rootstocks can be successfully grafted only during a relatively narrow range of development (Navarro *et al.*, 1975) and optimal conditions may differ for different rootstock cultivars (Navarro, 1981). The cultural conditions of scion stock plants and grafted plants also affect grafting success.

A technique termed minigrafting, in which tissue culture-derived material is grafted on to seedling rootstocks *in vivo*, has also been investigated (De Pasquale *et al.*, 1999; Yang *et al.*, 2000). Shoots, roots, inverted roots and somatic embryos were grafted with varying degrees of success; success was very high with shoots.

Somatic embryogenesis from stigma and style tissue has been utilized to eliminate citrus psorosis virus from three citrus types (D'Onghia *et al.*, 2001).

5. Somatic Cell Genetics

5.1. Regeneration

De novo regeneration of *Citrus* was described almost 50 years ago (Stevenson, 1956; Maheshwari and Rangaswamy, 1958) and is the basis for applying modern genetics to this crop.

5.1.1 Somatic embryogenesis

Since many citrus types are polyembryonic and adventive embryos are produced *in vivo* from nucellar tissue, most studies on *in vitro* somatic embryogenesis have involved the culture of adventitious embryos, isolated nucelli or whole fertilized or unfertilized ovules. Embryogenic citrus cultures have been obtained from entire fertilized ovules or the nucellus of fertilized ovules (Maheshwari and Rangaswamy, 1958; Rangaswamy, 1961; Sabharwal, 1963). Kochba *et al.* (1972) obtained embryogenic callus from unfertilized ovules of 'Shamouti' orange in which there was no evidence of nucellar or zygotic embryos (Spiegel-Roy and Kochba, 1973).

Induction. Induction of somatic embryogenesis in citrus has generally involved culture of ovules or nucelli. The ovules, fertilized or unfertilized, or isolated nucelli are cultured on Murashige and Skoog (1962) (MS) medium as modified by Murashige and Tucker (1969) (MT) for culture of citrus callus. Explants from monoembryonic and polyembryonic types respond differently.

Monoembryonic cultivars. Rangan *et al.* (1968, 1969) extracted nucelli from fertilized ovules of several monoembryonic cultivars 100–120 days after pollination. Induction of embryogenic cultures has also been reported from nucellar explants from fertilized ovules of other monoembryonic genotypes (Bitters *et al.*, 1970; Deidda, 1973). However, induction of embryogenic cultures from unfertilized ovaries and ovules and from nucelli excised from unfertilized ovules of monoembryonic genotypes has failed (Button and Kochba, 1977; Kobayashi *et al.*, 1982).

Polyembryonic cultivars. In contrast, direct induction of somatic embryos is possible from unfertilized ovules (Kobayashi *et al.*, 1982) and nucellar explants from abortive (Bitters *et al.*, 1970) and unfertilized (Button and Bornman, 1971; Mitra and Chaturvedi, 1972) ovules of polyembryonic citrus types (Kochba *et al.*, 1972; Button, 1977; Button and Kochba, 1977). Ovules or nucelli were obtained from flowers or immature fruit.

In monoembryonic and polyembryonic types, the somatic embryos appear to arise directly from the nucellus without an intervening callus phase. In early studies, kinetin and indoleacetic acid (IAA) were added to the induction medium, but medium supplemented only with malt extract is equally effective (Starrantino and Russo, 1980). Maltose may be superior to sucrose as a carbohydrate source for induction (Perez *et al.*, 1998a).

Starrantino and Russo (1980) cultured undeveloped ovules of several cultivars of navel oranges and lemon from ripe fruits 8–15 months after anthesis. Somatic embryos were induced directly from adventive embryos in the undeveloped ovules. Fruiting trees from embryos in these cultures have been obtained (Starrantino and Russo, 1983). This approach has succeeded with polyembryonic citrus types, including sweet orange, grapefruit, mandarins and lemons, so that embryogenic cultures can be initiated throughout much of the year (Moore, 1985; Gmitter and Moore, 1986).

Embryogenic cultures have also been induced from pistil tissue (Carimi *et al.*, 1994, 1998b, 1999). Matsumoto and Yamaguchi (1983) induced somatic embryos and adventitious buds from the stems of *in vitro*-grown nucellar seedlings of trifoliate orange, and this is the only report of embryo production from a non-floral tissue.

Maintenance. Embryogenic cultures can proliferate on semi-solid medium containing 5.71 μM IAA and 4.65 μM kinetin, and form green embryos that develop to maturity (Kochba and Spiegel-Roy, 1977a). Malt extract and low concentrations of adenine enhanced somatic embryogenesis, whereas other kinetin : IAA ratios and high adenine

concentrations suppressed it (Kochba and Spiegel-Roy, 1977b). Secondary embryos are produced from these adventive embryos (Kochba *et al.*, 1972; Chaturvedi and Mitra, 1974; Juarez *et al.*, 1976; Button and Kochba, 1977), frequently from the hypocotyl of existing embryos. A friable embryogenic culture can also be produced, which consists entirely of small globular proembryos at various stages of development, i.e. proembryonal masses (PEMs) (Button *et al.*, 1974), which arise from single cells.

The establishment and maintenance of PEMs in culture are quite arbitrary. After several subcultures, embryogenic cultures become habituated (Spiegel-Roy and Kochba, 1973; Kochba and Spiegel-Roy, 1977b). The PEMs either cease proliferating or they lose embryogenic competence. Therefore cultures are usually stressed in order to restore embryogenic competence: (i) infrequent subculture; (ii) deletion of sucrose from the culture medium (Kochba and Button, 1974); (iii) replacement of sucrose by another sugar (Kochba *et al.*, 1978). Irradiation of embryogenic cultures can stimulate PEM proliferation (Kochba and Spiegel-Roy, 1977b). This has also been observed with low levels of some antibiotics (Moore *et al.*, 1988). Studies have continued on factors that influence growth and differentiation of embryogenic cultures (Cabasson *et al.*, 1997; Chapman *et al.*, 2000; Ikeda *et al.*, 2000; Jimenez *et al.*, 2001); the development of synchronized, proliferating cultures is a problem.

Development/maturation. Because of the habituated nature of PEMs, removal of growth regulators from medium does not necessarily result in embryo development and maturation. Rather this occurs within the proliferating masses. Some of the developing embryos appear to be normal, but others are incomplete or malformed. Thus, maturation generally occurs on induction and maintenance medium. Niedz and Bauscher (2001) have recently shown that the use of certain semi-permeable membranes (e.g. cellulose acetate) can positively affect the normalizing of somatic embryogenesis, thereby improving the efficiency of plant

recovery. Recently, we have had good success with many genotypes (including several mandarins and lemons) using an MT basal medium supplemented with 23.23 μM sucrose and 0.5 g/l malt extract (J.W. Grosser, unpublished data).

Germination. Mature somatic embryos can germinate spontaneously, but are often transferred to MT medium containing 2.89 μM gibberellic acid (GA_3) (Moore, 1985). A variable percentage of somatic embryos will germinate normally and develop into plants that can be transferred to soil. Finally, there has been some research on encapsulating somatic embryos of *C. reticulata* and sweet orange (Antonietta *et al.*, 1998; Nieves *et al.*, 1998).

5.1.2. Organogenesis

Induction. Shoots have been induced from cultures of different *Citrus* spp. on modified MS medium (Chaturvedi and Mitra, 1974; Raj Bhansali and Arya, 1978; Goh *et al.*, 1995; Normah *et al.*, 1997). Explants have included shoot meristems of seedlings and mature trees (Barlass and Skene, 1982; Paudyal and Haq, 2000; Kotsias and Roussos, 2001), stem sections (Grinblat, 1972; Chaturvedi and Mitra, 1974; Raj Bhansali and Arya, 1978, 1979; Barlass and Skene, 1982; Moore, 1986; Perez-Molphe-Balch and Ochoa-Alejo, 1997; Ghorbel *et al.*, 1998; Garcia-Luis *et al.*, 1999; Moreira-Dias *et al.*, 2000, 2001), root sections (Raj Bhansali and Arya, 1978), root meristems (Sauton *et al.*, 1982) and leaf sections (Chaturvedi and Mitra, 1974). Maximum shoot production has been achieved with 1.11–22.19 μM benzyladenine (BA). Adventitious shoots have been induced directly from stem segments without a callus phase (Barlass and Skene, 1982) or following callus initiation and proliferation (Chaturvedi and Mitra, 1974; Raj Bhansali and Arya, 1979). Auxin and malt extract in the medium are usually beneficial or essential for shoot production (Chaturvedi and Mitra, 1974; Raj Bhansali and Arya, 1978, 1979). The DBA3 shoot induction medium (Deng *et al.*, 1992) is effective for organogenesis from numerous explants, including roots,

somatic embryos and seedling internodes. DBA3 is semi-solid MT basal medium supplemented with 0.045 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 13.31 μM BA, 1.5 g/l malt extract and 20 ml/l coconut water.

Development. There are no specific conditions for growing out the adventitious shoots formed; they enlarge in size sufficiently for rooting in the media described above.

Rooting. Rooting of excised shoots has been accomplished on several auxin-based medium formulations, including RMAN medium (half-strength MT basal medium containing 0.02 mg/l naphthaleneacetic acid (NAA) and 0.5 g/l activated charcoal) (Grosser and Gmitter, 1990).

5.1.3. Haploid recovery

Hidaka *et al.* (1979) and Chen *et al.* (1980) recovered haploid, diploid and mixoploid plantlets from *Poncirus* and *Citrus* anthers, respectively. Anthers from sour orange and sweet orange yielded only diploid regenerants (Hidaka *et al.*, 1982; Hidaka, 1984), as did lime anthers (Chaturvedi and Sharma, 1985). Hidaka and Omura (1989) presented histological evidence to describe various routes of microspore development in cultured *Citrus* and *Poncirus* anthers, leading in some instances to multinucleate cellular structures and haploid embryo development. Nuclear fusions that were sometimes observed within these structures were suggested as the cause of diploidy in regenerants. As tetraploid plants ($2n = 4x = 36$) are produced in greater abundance in breeding programmes, it may be feasible to culture their anthers to produce useful diploids ($2n = 2x = 18$).

5.1.4. Triploid production

Mandarins and mandarin hybrids are among the most important fresh fruit citrus types, but many are characterized by the production of seedy fruit. Consumer demands for seedless, easy-to-peel fruit are increasing. Therefore, *in vitro* methods have been developed to produce tetraploids for use in breed-

ing programmes and, subsequently, triploids from $2x \times 4x$ crosses. Two pummelo-grapefruit triploid hybrids, 'Melogold' and 'Oroblanco', have been patented and released (Soost and Cameron, 1980, 1985). The very promising seedless triploid mandarin hybrid, 'Tacle', produced from a cross of 'Clementine' mandarin with tetraploid 'Tarocco' sweet orange, was recently released for commercial production in Italy (Reforgiato Recupero and Tribulato, 2001). Although autotetraploids have been found among nucellar seedlings of the major cultivar groups (Cameron and Frost, 1968), the full exploitation of this interploidy breeding approach has been limited in the past by the lack of suitable tetraploids available for use as breeding parents. However, many more quality tetraploid breeding parents are now becoming available, resulting from somatic hybridization (Tusa *et al.*, 1996; Kobayashi *et al.*, 1997; Del Bosco *et al.*, 1999; Guo and Deng, 1999, 2000; Grosser *et al.*, 2000a,b) and other techniques, including induction by colchicine treatment of axillary buds *in vivo* (Barrett, 1974), *in vitro* treatment of undeveloped ovules and embryogenic callus (Gmitter and Ling, 1991; Gmitter *et al.*, 1991) and a combination of colchicine treatment and micrografting (Oiyama and Okudai, 1986). In the past, $4x \times 2x$ crosses have been more successful for producing triploids than the reciprocal because of a more favourable embryo: endosperm ploidy ratio (3:5 as opposed to 3:4) (Esen and Soost, 1973; Soost and Cameron, 1975), resulting in the recovery of normally developed seeds and the ability to plant them directly in soil. Thus, monoembryonic zygotic tetraploids are desired as female parents; however, most previously available tetraploid clones are polyembryonic and produce few or no zygotic seedlings.

This problem can be addressed by rescuing *in vitro* zygotic triploid embryos from monoembryonic (and in some cases polyembryonic) diploid parents crossed with tetraploid males. Starrantino and Reforgiato-Recupero (1981) cultured globular to heart-shaped zygotic embryos that were excised from underdeveloped seeds from fruit 3–4 months after pollination of diploid monoem-

bryonic clones with pollen of tetraploid clones. Plants recovered using their method were triploid. Oiyama *et al.* (1991) were also able to recover triploid plants by culturing undifferentiated embryos excised from poorly developed seeds of mature fruit resulting from a monoembryonic diploid seed parent crossed with pollen from a tetraploid *Citrus* + *Poncirus* somatic hybrid. Embryo rescue is now being widely used to recover triploids from interploidy crosses for seedless mandarin improvement (Grosser *et al.*, 2000a,b) and for lemon/lime improvement (Tusa *et al.*, 1992; Chandler *et al.*, 2000; Vilorio *et al.*, 2001). The UF citrus breeding programme routinely produces between 1000 and 3000 triploid hybrids annually, using embryo rescue techniques (F.G. Gmitter, unpublished data), and similar activities are taking place elsewhere. When female parents are polyembryonic, flow cytometry is used to efficiently separate triploid progeny from diploid nucellar types, prior to planting in soil (Vilorio *et al.*, 2001).

Triploid plants can also be produced directly by regeneration from endosperm (Gmitter *et al.*, 1990). Induction of triploid embryogenesis requires GA₃ and doubled mineral nutrient concentrations in the medium. However, this approach is tedious and genotype-specific, and it is impractical for triploid breeding and seedless cultivar development.

Fusion of diploid somatic protoplasts with haploid gametic protoplasts is another route to triploidy. Ollitrault and co-workers have produced more than 100 triploid hybrids from the somatic hybridization of two haploid cell lines of 'Clementine' mandarin with 11 other selected diploid cultivars, including mandarins, sweet oranges, grapefruit, lime and kumquat (Ollitrault *et al.*, 1996a; Grosser *et al.*, 2000b). Triploid progenies produced using this approach should display limited recombination and polymorphism, based on recombination events that occurred prior to establishment of the haploid cell line. This could potentially improve selection efficiency by improving overall population quality, because the diploid selection genotype would undergo neither segregation nor

recombination. The tradeoff for this is the more limited polymorphism upon which to impose selection, and the procedure is limited by the availability of haploid embryogenic cell lines.

A fairly simple approach has been used to recover triploid hybrid seedlings from diploid $\times$ diploid crosses. Certain monoembryonic diploids produce occasional unreduced gametes, which result in triploid zygotes upon fertilization (F.G. Gmitter, unpublished data). The triploid zygotic embryos are found within undeveloped, shrivelled seeds, and they can be recovered by standard embryo rescue techniques. These same monoembryonic diploids also produce some tetraploid offspring following interploid hybridization, and frequently these tetraploids arise from normally developed seeds. Such tetraploids can be utilized in subsequent breeding cycles.

5.1.5. Protoplast isolation and culture

Isolation and culture of citrus protoplasts from embryogenic cultures, followed by somatic embryo development, were first reported by Vardi *et al.* (1975). 'Shamouti' sweet orange plants were regenerated from protoplasts isolated from embryogenic cultures (Vardi, 1977). These techniques were subsequently extended to several other citrus cultivars (Vardi *et al.*, 1982). Citrus protoplasts have been isolated from many other explants, including non-morphogenic calluses, leaves and pollen tetrads (Grosser and Gmitter, 1990). Plants of citrus cultivars (Grosser, 1994), somatic hybrids (Kobayashi and Ohgawara, 1988; Ohgawara *et al.*, 1989; Grosser and Gmitter, 1990; Grosser *et al.*, 2000b; Guo and Deng, 2001), somatic cybrids (Ollitrault *et al.*, 1996b; Alonzo *et al.*, 2000; Grosser *et al.*, 2000b; Moreira *et al.*, 2000a) and citrus relatives (Jumin and Nito, 1996) have been regenerated. Generally, highly buffered mixtures of cellulase, macerase and often pectolyase are used to isolate citrus protoplasts (Grosser and Gmitter, 1990). Citrus protoplast culture media range from simple high osmoticum basal medium (MS or MT) to the complex BH3 medium (Grosser and Gmitter, 1990). Protoplasts have

been successfully cultured in thin-layer liquid culture (Grosser and Gmitter, 1990) and embedded in agarose or calcium alginate beads (Niedz, 1993). The totipotency of citrus protoplasts has resulted in many successful applications of citrus protoplast technologies.

5.2. Genetic manipulation

5.2.1. Mutation induction and somaclonal variation

Breeding objectives. The objective is to induce a change in one or more characteristics of the fruit or tree of an otherwise acceptable cultivar. Most frequently, the change sought by induced mutation breeding through irradiation is a reduction in seed number without perturbation of other desirable characteristics. Induced mutations, resulting in the reduction or near complete elimination of seeds, have been associated with chromosomal aberrations, but not with single gene mutations (Gmitter *et al.*, 1992). The objectives sought when attempting to exploit somaclonal variation are much more generalized and relate to any of the traits considered important for cultivar improvement of primarily those citrus cultivar groups for which hybridization is an impractical approach.

Protocol. The techniques for inducing mutations by irradiation were well documented many years ago, so they are not presented here (Russo *et al.*, 1981; Hearn, 1984, 1986; Spiegel-Roy *et al.*, 1985). The methods of somaclonal variation are those of *in vitro* techniques described elsewhere in this chapter, followed by rigorous evaluation of field performance of regenerants.

Accomplishments. The induction and/or selection of useful variation by mutagenesis of seeds or axillary buds is a particularly attractive approach to *Citrus* cultivar development, particularly for hybrid citrus types such as sweet orange and grapefruit, which are not amenable to sexual hybridization. The first commercially significant cultivar from a mutation breeding programme was

the 'Star Ruby' grapefruit, which arose from irradiated seed of 'Hudson' grapefruit. 'Star Ruby' was selected on the basis of deep red flesh colour and reduced seediness (Hensz, 1977). Seedless clones of normally seedy 'Pineapple' orange, 'Duncan' and 'Foster' grapefruit (Hearn, 1984, 1986), 'Monreal' clementine mandarin (Russo *et al.*, 1981) and 'Eureka' lemon (Spiegel-Roy *et al.*, 1985) have been selected from irradiated seed or axillary bud-derived plants.

A long-term investigation of potential exploitation of somaclonal variation for citrus cultivar improvement has been under way during the past 15 years to identify useful variants regenerated by somatic embryogenesis, organogenesis or protoplast-to-plant regeneration methods, with the focus on 'Hamlin' and 'Valencia' sweet oranges (Grosser *et al.*, 2001a). Significant stable variation has been observed for tree and fruit characteristics from all of these regeneration pathways, with 'Valencia' showing more profound and useful variation. Significant variation has been observed for the following traits: (i) tree characteristics, including canopy size and shape, leaf size and shape, ploidy level and juvenility, thorniness and vigour; and (ii) fruit characteristics, including (a) brix : acid ratio; (b) colour of fruit and juice; (c) maturity date; (d) size; (e) rind thickness; (f) rag; (g) juice content; and (h) seediness. Based on fruit quality analyses over several seasons, several clones have been selected and propagated for further study. Selected clones for processing include early (1–2 months earlier) and late (1–2 months later) maturing 'Valencia' somaclones with superior quality, which should facilitate the processed 'not from concentrate' (NFC) juice industry, and clones of standard maturity with higher soluble solids. Improved 'Hamlin' clones have been selected for earlier maturity, better juice colour and increased soluble solids. 'Valencia' somaclones with fresh-market potential have also been identified, including seedless clones, along with clones exhibiting a more melting flesh than standard 'Valencia'. These results indicate that the generation and evaluation of somaclonal variation is a useful method

for developing improved citrus cultivars, especially for species recalcitrant to conventional breeding such as sweet orange and grapefruit.

Spontaneous, naturally occurring somatic mutations may be recovered in whole plants produced from undeveloped ovules and seeds of chimeric sector fruit, using *in vitro* regeneration methods. The phenomenon of fruit sector chimeras, observed among most citrus cultivars (Bowman *et al.*, 1991), may result from the expression of somatic genetic mutations or somatic segregation (Cameron and Frost, 1968). Some of the phenotypic changes observed include increased pigmentation, earlier or later maturity and differences in susceptibility to pest-induced rind damage. Iwamasa *et al.* (1977) recovered plants producing fruit with either entirely yellow or orange rinds from seed extracted from a sector 'Fukuhara' sweet orange with normal colour and a yellow mutant sector. Bowman *et al.* (1991) recovered tetraploid plants from seed taken beneath gigas (presumably tetraploid) rind sectors of 'Valencia' orange and 'Orlando' tangelo fruit. Because the L-II histogenic layer in *Citrus* gives rise to the rind tissues, excluding the thin epidermis, as well as to gametes and the nucellus (Soost and Cameron, 1975), it should be possible to recover plants that possess the genetic mutations observed in rind tissue in all three histogenic layers, thus providing a valuable source of genetic variants, with potential as improved cultivars. Preliminary evaluations of fruit quality characteristics from trees produced either from undeveloped ovules cultured *in vitro* or seeds taken from mutant sectors have revealed phenotypic changes among the offspring. The original mutant phenotype was not recovered in the offspring.

There are a few reports of *in vitro* selection in citrus. Efforts have been made to select embryogenic lemon callus that is resistant to *Phoma tracheiphila* (Gentile *et al.*, 1992, 1993). The use of a *Phytophthora* culture filtrate to select resistant types has been investigated (Vardi *et al.*, 1986). Embryogenic cell lines of *P. trifoliata* that were tolerant of increased levels of NaCl were produced (Beloualy and Bouharmont, 1992).

5.2.2. Somatic hybridization

Breeding objectives. Production of somatic hybrids and cybrids among elite citrus selections is routine, and plants have been regenerated from > 200 parental combinations (Vardi *et al.*, 1987, 1989; Grosser *et al.*, 2000b). The primary strategy for scion improvement is to produce allotetraploid parents for use in interpollid crosses, by combining complementary elite scion cultivars (Grosser *et al.*, 2000a,b). Fertile tetraploid somatic parents will facilitate seedless triploid development. Another strategy for scion improvement is the direct production of triploids by haploid + diploid fusion (Ollitrault *et al.*, 1997, 1998a; Grosser *et al.*, 2000b). The primary strategy for rootstock improvement has been to produce somatic hybrids of complementary rootstock parents that have potential for improved disease resistance, tree size control and horticultural performance (Grosser and Gmitter, 1990; Grosser *et al.*, 1996, 2000a,b; Ollitrault *et al.*, 1998b; Grosser and Chandler, 2000, 2001a). Such hybrids have potential as rootstock and can also be used for breeding and selection at the tetraploid level to maximize genetic diversity in zygotic progeny (Grosser *et al.*, 2001b). Wide somatic hybrids of citrus are also possible, including combinations of citrus with sexually incompatible or difficult-to-cross citrus relatives (Louzada and Grosser, 1994; Grosser *et al.*, 1996, 2000b; Motomura *et al.*, 1997; Guo and Deng, 2001). Such wide hybrids may have limited utility as citrus rootstocks, but could broaden the germplasm base available for rootstock improvement. Production of citrus somatic cybrids is now possible, which may have applications for both scion and rootstock improvement (Tusa *et al.*, 2000).

Protocol. Citrus somatic hybrids are most commonly produced from the fusion of protoplasts isolated from embryogenic cultures of one parent with leaf-derived protoplasts of another parent. At least one parent in any fusion combination must be embryogenic to provide the capacity for plant regeneration in the somatic hybrid progeny. More than 100 parental combinations have been used to produce somatic hybrids with the following protocol at the UF CREC.

Embryogenic cultures should be maintained on semi-solid medium or as suspension cultures on either EME (MT basal containing 0.5 g/l malt extract) or H+H (modified MT basal containing 1.55 g/l glutamine, 0.5 g/l malt extract and 50% KNO₃ and NH₄NO₃). Embryogenic cultures should be in the log phase of growth. Approximately 1–2 g embryogenic culture is transferred into a 60mm × 15 mm Petri dish (approx. 2 ml suspension culture) and resuspended in 2.5 ml 0.7 M BH3 medium (Grosser and Gmitter, 1990). BH3 is nitrate-free MT basal medium containing 3.1 g/l glutamine, 20 ml/l coconut water and Kao and Michayluk (1975) organic addenda. Filter-sterilized enzyme solution (1.5 ml) containing 0.7 M mannitol, 12.0 mM CaCl₂, 6.0 mM methyl ethane sulphonate (MES) buffer, 1.4 mM NaH₂PO₄, 1% Onozuka RS cellulase, 1% Macerace, 0.2% pectolyase Y-23 (pH 5.6) is added. Dishes are sealed and incubated overnight on a rotary shaker at 2 rpm in low light or darkness. Optimum yields of leaf protoplasts are obtained using fully expanded leaves of new flushes that have not fully hardened, from seedlings or recently budded plants maintained in a growth chamber or heavily shaded greenhouse. Use of *in vitro*-grown leaf material precludes the need for disinfestation prior to isolation. Leaf material can be disinfested by brief immersion in 1 M HCl followed by a 12–15 min immersion in 10–15% commercial bleach containing three drops of surfactant, followed by a 5 min rinse and two 10 min rinses in double-distilled water. Leaf material is cut into thin strips with a sharp scalpel and incubated in 3 ml of enzyme solution combined with 8 ml 0.7 M BH3 medium in a 125 ml side-armed Erlenmeyer flask. Leaf material in the enzyme cocktail is evacuated for 15 min at 50 kPa to facilitate enzyme infiltration.

Following incubation, preparations are passed through a 45 µm mesh screen to remove undigested cell clumps and debris. Protoplast-containing filtrates are then centrifuged for 4–10 min at 100 g in 15 ml calibrated centrifuge tubes. The supernatant is removed, and the pellet containing the protoplasts is gently resuspended in 5 ml of

a 25% sucrose solution containing CPW nutrients (27.2 mg/l KH_2PO_4 , 100 mg/l KNO_3 , 150 mg/l CaCl_2 , 250 mg/l MgSO_4 , 2.5 mg/l $\text{Fe}_2(\text{SO}_4)_3$, 3.6 H_2O , 0.16 mg/l KI, 0.00025 mg/l CuSO_4 , pH 5.8) (Frearson *et al.*, 1973). A 13% mannitol solution (2 ml) (containing CPW salts) is slowly added directly to the sucrose layer followed by centrifugation for 6 min at 100 g. Protoplasts form a band at the interface between the sucrose and the mannitol, and are removed and resuspended in BH3 medium.

Electrofusion of citrus protoplasts has been described by Saito *et al.* (1991), Hidaka and Omura (1992), Ling and Iwamasa (1994), Ollitrault *et al.* (1996b) and Guo and Deng (1998). Equal volumes of purified protoplasts from each parental source are mixed in 0.6 M BH3 medium and centrifuged for 4 min at 100 g. The pellets are suspended in a volume of BH3 medium equal to four to 20 times the volume of the original pellet, and two drops of the resuspended mixture are pipetted into 60 mm $\times$ 15 mm plastic Petri dishes. Two drops of polyethylene glycol (PEG) solution (40% PEG 8000, 0.3 M glucose, and 66 mM CaCl_2 at pH 6) are added and the mixture is incubated for 8 min. Two drops of A + B solution (9 : 1 v : v, A = 0.4 M glucose, 66 mM CaCl_2 and 10% dimethyl sulphoxide (DMSO) at pH 6, and B = 0.3 M glycine at pH 10.5) are added. A + B solutions should be mixed just prior to use. After 12 min incubation, 12–15 drops of BH3 medium are added to the periphery of the protoplasts. After 5 min, PEG plus (A + B) solution is replaced with 15 drops BH3 medium. After incubating for 10 min, BH3 medium is replaced with 12–15 drops fresh BH3 medium. Repeat this washing step twice, carefully avoiding the loss of protoplasts. After the final wash, protoplasts can be cultured directly either in a shallow pool (eight to 12 drops medium) or thin-layer culture (1.5 ml medium) in BH3 medium, EMEP medium or a 1 : 1 mixture of BH3 and EMEP (Grosser and Gmitter, 1990). Plates are sealed and cultured either in darkness or with low light intensity.

Following incubation for 4–6 weeks, cultures are supplemented with medium containing reduced osmoticum, by addition of ten to 12 drops of a 1 : 2 mixture of 0.6 M

BH3 medium and 0.38 M EME medium (MT basal containing 125 g/l sucrose and 0.5 g/l malt extract). Two weeks later, cultures are transferred to semi-solid medium in Petri dishes with reduced osmoticum, i.e. 2 ml of a 1 : 2 mixture of BH3 medium and 0.15 M EME medium (MT basal containing 50 g/l sucrose and 0.5 g/l malt extract) in each dish. The contents are decanted on to semi-solid medium containing EME medium, and the protoplast-derived colonies are spread evenly over the entire plate. Cultures with low-frequency embryo development can be transferred to EME medium containing maltose instead of sucrose. Somatic embryos mature and germinate on medium for somatic embryo development (Grosser and Gmitter, 1990). Rooted plants can be transferred to any suitable commercial potting mixture and maintained under a high relative humidity for 2–3 weeks for acclimatization.

Somatic hybrids are identified and verified by morphological evaluation, isozyme or molecular marker analyses and ploidy evaluation. Flow cytometry is now the preferred method for ploidy analyses (Ollitrault *et al.*, 1996a). RAPD analysis is the preferred method for showing a nuclear contribution from both parents. Cytoplasmic genome analysis is commonly done by RFLP (Kobayashi *et al.*, 1991; Moreira *et al.*, 2000b) and, more recently, by cleaved amplified polymorphic sequence (CAPS) analysis (P. Ollitrault, personal communication).

Accomplishments. Somatic hybrids have been produced from > 200 parental combinations (see Grosser *et al.*, 2000b). Production of somatic hybrid allotetraploid breeding parents has greatly enriched the pool of tetraploid genitors available for use in inter-ploid breeding to produce seedless triploids (Tusa *et al.*, 1990; Grosser *et al.*, 1992, 1998a; Kobayashi *et al.*, 1995; Deng *et al.*, 1996; Mourao *et al.*, 1996; Ollitrault *et al.*, 1998a). It is expected that the higher quality of many of these hybrids will translate to a higher percentage of triploid progeny with good quality. At the UF CREC, many somatic hybrids have flowered and pollen of the following somatic hybrids has been used to

produce triploids: 'Nova' mandarin + 'Succari' sweet orange (Grosser *et al.*, 1998a); 'Rohde Red Valencia' sweet orange + 'Dancy' mandarin; 'Valencia' sweet orange + 'Murcott' tanger; 'Valencia' + ('Robinson' mandarin hybrid $\times$ 'Temple' tanger); 'Hamlin' sweet orange + ('Clementine' mandarin $\times$ 'Minneola' tangelo); 'Succari' sweet orange + 'Dancy'; 'Succari' + 'Page' tangelo; 'Succari' + 'Minneola'; and 'Pink Marsh' grapefruit + 'Murcott'. Several triploid plants have been produced (F.G. Gmitter, unpublished data), and many are under evaluation. Numerous triploid somatic hybrids produced by Ollitrault and co-workers via direct haploid + diploid fusion are also in the field for evaluation; the most promising are hybrids of haploid 'Clementine' with diploid 'Willow leaf' mandarin, 'Kinnow' mandarin, 'Shamouti' sweet orange and 'Star Ruby' grapefruit (see Grosser *et al.*, 2000b). Clearly, somatic hybridization will have a major impact on seedless, easy-peel mandarin improvement.

Numerous somatic hybrids produced by combining complementary rootstock parents have been produced and propagated, and are under evaluation in commercial rootstock trials. Citrus trees on somatic hybrid rootstocks generally produce smaller trees but with higher yield efficiencies (based on 1–3 years of yield and fruit quality data) than control trees on diploid rootstocks (Grosser and Chandler, 2001a). Therefore, somatic hybrid rootstocks may be useful for high-density plantings. A somatic hybrid of *C. deliciosa* + *P. trifoliata* produced by Ollitrault *et al.* (1998b) is showing promise, as it is immune to CTV and resistant to *Phytophthora* and shows a good performance on calcareous soils. Somatic hybrid rootstocks that are showing good potential in Florida and are amenable to standard nucellar seed propagation include sour orange + Carrizo citrange, sour orange + Palestine sweet lime, Cleopatra mandarin + *P. trifoliata*, Cleopatra mandarin + rough lemon and Cleopatra mandarin + Volkamer lemon (Grosser and Chandler, 2001a). Two other somatic hybrid rootstocks performing well in field trials are sour orange + Rangpur and 'Nova' mandarin + Hirado Buntan pummelo (zygotic

seedling). Although these latter two selections are not amenable to seed propagation, they are being used as females in crosses at the tetraploid level with other high-performance somatic hybrids to maximize genetic diversity in resulting progeny (Grosser *et al.*, 2001b). Progeny from such crosses have been screened for *Phytophthora* resistance and adaptation to high pH and calcareous soil, and several promising tetraploid zygotic hybrids have been identified for further study. Field performance information on many additional somatic hybrid rootstocks will be forthcoming, as several trials are maturing. Several wide intergeneric citrus hybrids have also been tested in rootstock trials, but their performance has been disappointing; however, two recently produced hybrids are promising, i.e. 'Succari' sweet orange + *Atalantia ceylanica* and 'Nova' mandarin + *Citropsis gilletiana* (Grosser *et al.*, 2000b). The real value of these wide hybrids may be as tetraploid breeding parents to further introgress useful genes from the related genera into more horticulturally useful forms, if they indeed prove to be fertile. Somatic hybrid rootstocks are expected to be available for commercial use in the near future, and they should facilitate a shift towards high-density plantings.

5.2.3. Genetic transformation

Genetic transformation of *Citrus* has made notable progress. The primary focus has been on transformation using genes with potential for disease resistance and for improved fruit quality. Most research has detailed the establishment of various transformation systems; *Agrobacterium*-mediated transformation has been the most successful technique. Various *Agrobacterium* strains have been used, and no consensus has been reached on the most effective strain for citrus (Gutierrez-E. *et al.*, 1997; Bond and Roose, 1998; Cevera *et al.*, 1998b; Ghorbel *et al.*, 2001a). Bond and Roose (1998) found that strain C58C1 was more effective in transforming sweet orange than either EHA101 or LBA4404, while Ghorbel *et al.* (2001a) found that EHA105, with the Ti plasmid from pTiBo542, was better for transformation of

several citrus types than a strain with a C58-based Ti plasmid. In most reports, a chimeric neomycin phosphotransferase II gene (*npt II*) with a nopaline synthase (*nos*) promoter has been used for selection on medium containing the antibiotic kanamycin sulphate. The scorable marker *uidA* (β -glucuronidase, GUS) has also frequently been utilized, most often with the cauliflower mosaic virus (CaMV) 35S promoter. For descriptions and comparisons of many of the *Agrobacterium* strains detailed below, see Hellens *et al.* (2000).

Breeding objectives. Many of the objectives for citrus cultivar improvement cannot be achieved by standard plant breeding methods, without resorting to molecular approaches. Despite a decades-long programme to introgress *Ctv*, the CTV resistance gene, from *P. trifoliata* into commercial citrus cultivars by sexual hybridization, there has yet to be an edible and commercially acceptable cultivar released. For this gene, and many others that have been or will be cloned, the opportunities for meaningful impact on cultivar improvements are entirely dependent on genetic transformation technology. The objectives to be targeted through genetic transformation are identical with the general objectives described above. Through a molecular approach, citrus cultivar improvement promises to be a more targeted and precise process, in comparison to the 'shot-gun' approaches that have been the historical precedent. It is important to recognize, however, that this molecular approach, albeit a powerful tool for specific improvement objectives, does not eliminate the utilization or reduce the value of the other plant breeding tools applied previously to the challenges of citrus cultivar development.

Protocol. The first reports of transformation in citrus entailed the introduction of DNA directly into protoplasts. Kobayashi and Uchimiya (1989) used PEG to induce uptake of plasmid pCT2T3 DNA containing *nptII* into 'Trovita' sweet orange protoplasts, although plants were not regenerated. Vardi *et al.* (1990) transformed rough lemon (*C. jambhiri* Lush.) protoplasts using a similar

procedure. The linearized plasmid used in these experiments, pCAP212, contained a dual, bidirectional promoter fragment of *Agrobacterium* T-DNA origin located between the coding sequences for chloramphenicol acetyltransferase (*cat*) and *nptII*. CAT activity detected in protoplasts 3 days after PEG treatment verified transient expression. Kanamycin did not reliably inhibit growth of non-transformed cells; however, paromomycin sulphate was effective. Rooted plants were verified as transgenic, and were the first transgenic citrus plants.

Niedz *et al.* (1995) regenerated transgenic sweet orange plants via electroporation of embryogenic protoplasts, and used green fluorescent protein (GFP) as a selectable marker (Niedz and McKendree, 1998). Fleming *et al.* (2000) transformed sweet orange protoplasts with a synthesized plasmid containing a GFP gene as a scorable marker via PEG-mediated uptake into embryogenic protoplasts followed by GFP selection of regenerating transgenic cells and embryos.

Yao *et al.* (1996) used particle bombardment to transform 'Page' tangelo ((*C. reticulata* Blanco $\times$ *C. paradisi* Macf. 'Minneola') $\times$ *C. reticulata* 'Clementine') suspension cultures with tungsten particles coated with a synthesized plasmid DNA that contained *nptII* and *uidA*. Many cells or small colonies were GUS-positive 1 day after bombardment, indicating transient gene expression, and up to 15 GUS-positive colonies for each bombardment were obtained after 8 weeks of culture on 100 mg/l kanamycin. PCR and Southern hybridization analyses verified transformation. The Southern blots revealed complex banding patterns, indicating DNA rearrangements and/or partial transfer, which is not uncommon with biolistic transformation. Although somatic embryos and plantlets were produced, no transgenic plants were analysed.

Hidaka *et al.* (1990) reported the first *Agrobacterium tumefaciens*-mediated transformation of *Citrus*. They inoculated embryogenic suspension cultures of 'Trovita' and 'Washington navel' sweet orange with an engineered *Agrobacterium* strain. Chimeric *nptII* and hygromycin phosphotransferase

(*hpt*) genes, both with a CaMV 35S promoter, were tested for selection. Proliferating cell colonies or embryos were obtained in some experiments and some plantlets were recovered; at least one plantlet that differentiated from a hygromycin-resistant somatic embryo was analysed by Southern analysis.

Moore *et al.* (1992, 1993) utilized *A. tumefaciens* EHA101 (pMON9793), containing *nptII* and *uidA*, to transform *Citrus* using organogenesis. Internodal stem pieces (approx. 1 cm long) were excised from 2- to 4-month-old *in vitro* citrus seedlings that had been grown in the light. The rootstock cultivar Carrizo citrange (*C. sinensis* (L.) Osb. × *P. trifoliata* (L.) Raf.) was used for many experiments because it is highly regenerable via organogenesis (Moore, 1986) and is polyembryonic, so that the seedlings are true to type (Moore and Castle, 1988). Swingle citrumelo (*C. paradisi* Macf. × *P. trifoliata*) and Key and Mexican lime were also used in some of these early experiments. Later experiments were performed with sweet and sour orange; these experiments were done using EHA101 (pGA482GG + the T36 CTV coat protein (CP) gene) (Gutierrez-E. *et al.*, 1997).

In early experiments, internodal stem segments were cultured vertically in semi-solid medium and inoculated with a drop of *Agrobacterium* suspension. Following the co-culture, the stem segments were transferred to regeneration/selection medium, usually containing 100 µg/ml kanamycin and various concentrations of BA. The segments were maintained in a vertical orientation and shoots were only produced from the portion of the segment that protruded from the medium, with little or no callus production. After 4 weeks, adventitious shoots were harvested and the segments were transferred to fresh selection medium. The regenerated shoots were immediately assayed for GUS activity by cutting small freehand sections from the basal ends of the shoots and assaying them histochemically for GUS (Jefferson, 1987). At 8 weeks, shoots were again harvested and the segments discarded. Transgenic plants were obtained with this method, but the technique was quite inefficient. Most of the shoots that regenerated on selection medium either were not trans-

formed or were chimeric (Gutierrez-E. *et al.*, 1997). The frequency of transformation varied with respect to species and cultivar. Transformation of Carrizo citrange with a gene construct containing the CP gene from CTV T36 (CTV-CP) (Sekiya *et al.*, 1991) was later described (Gutierrez-E. *et al.*, 1997), and several plants expressing the CTV-CP were generated. The protein was expressed at varying levels in different transgenic plants; however, since Carrizo is resistant to CTV, these plants could not be challenged by virus inoculation. Transformation experiments were also carried out with the CP gene and Key lime, sour orange and pineapple sweet orange, all of which are susceptible to CTV. Many GUS-positive shoots were produced from Key lime; however, the shoots were difficult to root and few transgenic plants were recovered. Few sour orange and pineapple sweet orange shoots could be regenerated using this procedure, thereby restricting the application of this transformation method.

Kaneyoshi *et al.* (1994) reported highly efficient transformation of *P. trifoliata*, the trifoliolate orange, using a similar technique and pBI-based vector plasmids that contained *nptII* and *uidA*. However, there were some important differences: (i) *in vitro*-germinated seedlings were grown for a shorter time (20 days) in darkness; (ii) epicotyl segments were excised from etiolated seedlings; (iii) acetosyringone (100 µM) was used as an activator before and during co-cultivation; (iv) the bacterial density for inoculation was greater, i.e. 5×10^8 cfu/ml, and segments were exposed to *Agrobacterium* for 15 min; (v) segments were cultured horizontally on selection medium; and (vi) for selection for NPT II expression and regeneration, segments were first cultured on MS medium containing 22.19 µM BA with 0.54 µM NAA and 100 mg/l kanamycin. Following shoot regeneration, the segments were transferred to medium with 2.22 µM BA and 200 mg/l kanamycin. Kaneyoshi *et al.* (1994) reported that 45% of inoculated segments regenerated shoots and up to 88% of the regenerated shoots were GUS positive. GUS-positive shoots rooted on semi-solid MS medium with 2.15 µM NAA at a frequency of 81%. Presence of T-DNA was confirmed in

regenerated plants by PCR and Southern analyses. More than 100 transgenic plants could be recovered in 2–3 months. This procedure with few modifications has been adapted for high-frequency recovery of transgenic grapefruit plants using EHA101 (pGA482GG) (Luth and Moore, 1999) or C58C1 (pBin35SGUS) (Yang *et al.*, 2000), both of which also contain *nptII* and *uidA* (Fig. 19.1.1).

Peña *et al.* (1995a) have reported high-efficiency transformation of the *Poncirus* × sweet orange hybrid Carrizo citrange. Internodal stem segments were excised from 5-week-old seedlings, and were inoculated with *Agrobacterium* EHA105 (p35SGUSINT) (4×10^7 vs. 4×10^8 cfu) and incubated on a roller drum. After co-cultivation, the segments were cultured horizontally on medium containing 100 mg/l kanamycin and 13.31 μ M BA, and maintained in darkness for 8 weeks, during which time shoots began to develop. The segments were then transferred to a 16 h photoperiod. After 4 weeks, there was no transformation; however, after culture for 1–2 additional months, shoots developed from stem callus, and these shoots were GUS positive at a high frequency (55%). Chimerism based upon GUS staining was common (24% of regenerated shoots); transformation was confirmed by PCR, Southern and Northern analyses. Peña *et al.* (1995a) micrografted tips of transformed shoots on to Troyer citrange with 100% survival. The grafted plants were subsequently grafted on to rough lemon to promote rapid growth. Approximately 47% of transgenic shoots were morphologically abnormal, which was possibly due to the prolonged culture period and a callus phase. Moreover, 5–6 months were required to produce transgenic shoots.

Peña *et al.* (1995b) produced transgenic sweet orange 'Pineapple' plants using this procedure with stem segments from greenhouse-grown 6- to 12-month-old seedlings. Segments were inoculated with drops of bacterial suspension and cultured vertically. In contrast to Carrizo citrange, only 8% of the regenerated shoots were GUS positive. Transgenic shoots could not be rooted, but were successfully micrografted. Bond and

Roose (1998) achieved the best transformation frequencies with C58C1 (p35SGUSINT) and 3-week-old epicotyl segments of 'Washington' navel orange, but the genetically similar 'Valencia' sweet orange could not be transformed.

Peña *et al.* (1997) again used EHA105 (p35SGUSINT) but varied the co-cultivation procedure to recover transgenic Key lime. Stem pieces from 6–12-month-old greenhouse-grown plants were co-cultivated with engineered *Agrobacterium* on feeder plates consisting of tomato suspension culture cells layered on medium containing relatively high levels of several auxins. The explants were then transferred to selection/regeneration medium and maintained in darkness for 2 weeks. Organogenic callus proliferated on the stem explants, and 14 GUS-positive and two chimeric shoots were produced from 304 explants. Shoot tip grafting of regenerated shoots on Troyer citrange seedlings was 100% successful, and the presence and expression of the transferred genes in the regenerants plants were verified by NPTII enzyme-linked immunosorbent assay (ELISA), PCR and Southern analyses.

Cevera *et al.* (1998c), using the same vector system, examined several other factors that affected transformation efficiency of Carrizo citrange. Although transformation efficiency was maximum after 5 days of co-cultivation, regeneration efficiency was lower because of *Agrobacterium* overgrowth. Therefore, co-cultivation periods of 2–3 days were considered optimal. Addition of 100 μ M acetosyringone to the co-cultivation medium increased transformation twofold. The positive effect of feeder plates on transformation was determined to be caused by the high auxin levels in the medium, and not by the tomato cells or filter paper layer (see above). Preculture of explants before co-cultivation negatively affected transformation efficiency. Selection medium containing 13.31 μ M BA promoted optimum regeneration, and 100 mg/l kanamycin was optimum for selection. Exposure of explants to darkness for 4 weeks during selection increased the regeneration of transgenic shoots and decreased the number of escape shoots produced. A transformation efficiency of 41%

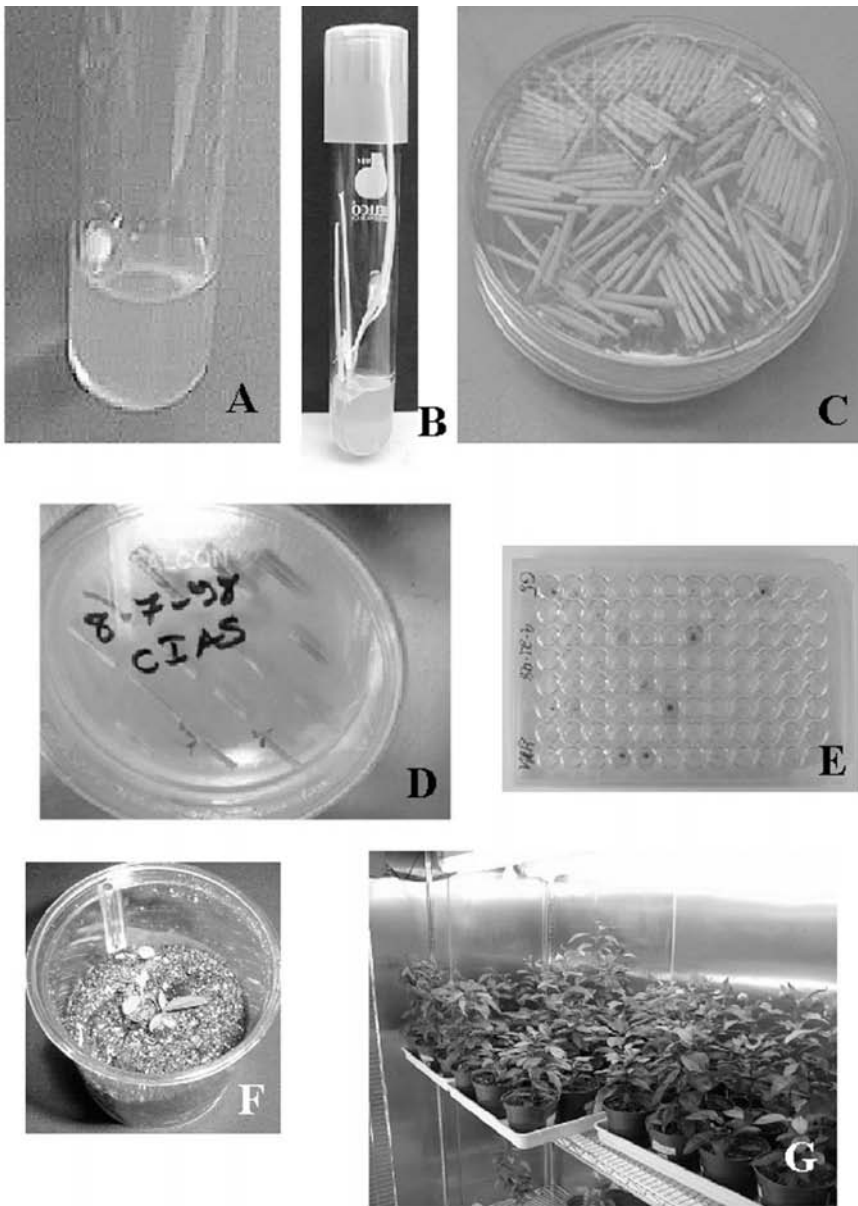


Fig. 19.1.1. Steps used for *Agrobacterium*-mediated transformation in the Moore laboratory. A. Seeds of the desired genotype are harvested from fruit, sterilized and placed in individual tubes on half-strength MS medium. B. The seeds are germinated in the dark such that they are etiolated and lack chlorophyll. C. Epicotyl sections are cut from the germinated seedlings aseptically, transferred to a prepared solution containing *Agrobacterium*, blotted to remove excess bacteria and co-cultured on medium without a selective agent for 2 days. D. Upon transfer to selective medium, which typically contains kanamycin as a selective agent and the appropriate level of BA for regeneration of the genotype being used, shoots are regenerated. E. Regenerating shoots are harvested individually and a subsection of their stem is assayed for GUS activity. F. GUS-positive shoots are typically rooted, first on medium and then transferred to sterile soil. G. After further development, the plants are transferred to pots, maintained first in a growth room under low light, and then transferred to the greenhouse. The plants pictured here are large enough for analyses by PCR, Southern, Northern and/or Western blotting, as appropriate.

was achieved using the optimized transformation procedure. Ghorbel *et al.* (1999) used this procedure with *Agrobacterium* EHA105 (pBin 19-*sgfp*), which contains a gene for the expression of GFP, to study transformation in three citrus types. GFP expression was localized in callus cells that formed from the cambium at the cut ends of explants after culture in darkness. Escape shoots did not differentiate from callus; therefore they concluded that callus formation from the cambium is essential for transformation. Ghorbel *et al.* (2000) used this procedure (with GUS produced from pBI121 as a scorable marker) to increase the transformation efficiency of sour orange, which has been difficult to transform. Plant age and condition were important factors; explants from 4-month-old greenhouse-grown seedlings performed better than those of older or younger seedlings. A similar technique was used to transform key lime (Dominguez *et al.*, 2000). In both of these cases, the pBI121-derived construct used for transformation contained the CTV-CP gene. When 42 transgenic lime plants were analysed, all of them contained at least one copy of the CTV-CP transgene, and 70% of them had multiple linked T-DNAs arranged at one locus. No correlation was found between CP expression and CTV-CP copy number or integration pattern.

Yu *et al.* (2002) further refined *Agrobacterium*-based transformation. Epicotyl segments from etiolated 2-week-old seedlings were cut in half longitudinally to increase the wounded area of the explants. This resulted in enhanced shoot differentiation with or without *Agrobacterium* inoculation. Optimization of inoculum density for each cultivar had significant effects on regeneration and transformation efficiencies. Explant preparation alone increased transformation frequency from 0.7% to 4.3% for sweet orange and from 5% to 40% for Carrizo citrange. Decreasing inoculum density resulted in increased transformation frequency from 2% to 8% for sweet orange and from 10% to 40% for Carrizo citrange. Optimizing 2,4-D concentration in the medium had a similar effect on transformation efficiency, and was cultivar-specific. Whole plant recovery was possible when

shoots > 2 mm in length were grafted on to rootstock seedlings *in vitro*. Transgenic shoots have been grafted on to etiolated seedlings grown *ex vitro* with survival as high as 100%, with plants showing more rapid growth following acclimatization than those grafted on *in vitro* seedlings (F.G. Gmitter, unpublished data).

In the most thorough analysis of transgenic citrus plants yet published, 70 transgenic Carrizo citrange plants from various experiments were characterized (Cevera *et al.*, 2000). All of the plants were nucellar in origin and 66 plants were diploid and four were tetraploid. Transgenic plants maintained consistent GUS expression over 5 years of analysis, although at different levels and with fluctuation over seasons in individual plants. T-DNA rearrangements, probably multiple inserts at a single locus, were detected in 11 of the 30 plants analysed and copy number ranged from one to six or more. A significant tendency to low GUS expression was associated with the presence of more than two T-DNA copies, but expression levels were highly variable in plants having fewer than two copies.

All of the transgenic plants obtained in the experiments described above were juvenile. Two approaches for transforming mature tissue have been reported. Cevera *et al.* (1998a) utilized mature sweet orange tissue that had been partially rejuvenated by grafting buds on to seedling rootstock. Internodal stem pieces were cut from flushes from these buds, inoculated with an *Agrobacterium* EHA105 (p35SGUSINT) and co-cultivated on tomato suspension culture cell feeder plates. After selection for 15 days in darkness, cultures were transferred to light and shoot regeneration was obtained after 2–5 months. Sixteen of the regenerated shoots were transgenic and some of these flowered; fruit were produced after just 14 months. Carrizo citrange was transformed to express constitutively either the *Arabidopsis* *LEAFY* (*LFY*) or the *APETALA1* (*API*) genes, which promote flower initiation (Peña *et al.*, 2001). Although plants expressing *LFY* displayed an abnormal phenotype, plants expressing *API* had fertile flowers and bore fruit as early as the first year.

Finally, in a strikingly different method of *Agrobacterium*-mediated transformation from those reported above, Perez-Molphe-Balch and Ochoa-Alejo (1998) reported high-frequency transformation following inoculation of Mexican lime stem segments with a wild-type *Agrobacterium rhizogenes* strain containing a binary vector plasmid pESC4 that contained *nptII* and *uidA*. The segments either produced shoots directly or produced roots from which shoots could be regenerated.

Accomplishments. There have been few reports of transgenes inserted other than marker genes. Several groups have transformed with the CTV-CP gene (Gutierrez-E. *et al.*, 1997; Dominguez *et al.*, 2000; Ghorbel *et al.*, 2000) or an untranslatable version of this gene (Yang *et al.*, 2000). When the p23 protein from this virus was used for transformation into Mexican lime plants, the plants displayed symptoms similar to those caused by the virus itself (Ghorbel *et al.*, 2001b). Cevera *et al.* (2000) have transformed Carrizo citrange with a gene, HAL2, isolated from yeast and implicated in salt-tolerance mechanisms. Yang *et al.* (2000) have also transformed grapefruit with the *Galanthus nivalis* agglutinin gene, a plant-derived insecticidal gene. Presence and expression of these genes was confirmed, but further characterization of plants as to the horticultural benefits of these genes has not been published. However, when a tomato pathogenesis-related protein PR-5 was constitutively expressed in transgenic sweet orange, one plant had increased tolerance of *Phytophthora citrophthora* (Fagoaga *et al.*, 2001).

5.3. Cryopreservation

Citrus conservation *in vitro* has involved sequential culture of nodal stem segments

(Marin and Duran-Vila, 1991). Cryo-preservation of embryogenic callus can be used for long-term storage of totipotent lines and citrus genetic resources. Cultures are cryoprotected with 10% (v/v) DMSO, frozen by slow cooling, stored in liquid nitrogen and warmed by fast thawing, after which they proliferate normally. Cryo-preserved embryogenic cultures have been shown to be suitable for further proliferation (Perez *et al.*, 1998b) and as a source for protoplast isolation, somatic hybridization and plant regeneration (Olivares-Fuster *et al.*, 2000).

6. Conclusions

The development of biotechnological methods of genetic manipulation has provided many new opportunities for *Citrus* cultivar improvement and development. The impediments to genetic progress described above, including barriers to sexual hybridization, the hybrid nature of important cultivated types and the scarcity of genetic information, can be addressed and potentially mitigated by these techniques. The greatest derived value from biotechnological manipulation of *Citrus* will be realized when these developments are integrated with a broad-based breeding and genetics programme directed towards overall germplasm enhancement, i.e. breeding parent and cultivar development.

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20

Sapindaceae

The *Sapindaceae* consist of a large group of tropical and subtropical species (approx. 2000 species in 140 genera) of largely Old World origin (Watson and Dallwitz, 1992 onwards). There are several tree species of economic importance within the family, and these include a number of tropical and subtropical fruits, including litchi (*Litchi chinensis* Sonn.), longan (*Dimocarpus longan* Lour.), akee (*Blighia sapida*), the Spanish lime (*Melicoccus bijugatus* Jacq.) and several *Nephelium* species. The latter genus consists of 22 species, which originated in South-east Asia, probably in western Malesia (Seibert, 1992; van Welzen and Verheij, 1992). *Nephelium lappaceum* L., the rambutan, is the most widely grown tropical fruit tree in this genus and is a major crop in

Thailand, Malaysia, Indonesia and elsewhere. Other closely related *Nephelium* species that are much appreciated for their fruit in South-east Asia but which are fairly underexploited include: *N. cuspidatum*, *N. hypoleucum*, *N. maingayi*, *N. ramboutan-ake* and *N. uncinatum* (Seibert, 1992). Although the rambutan has a chromosome number of $2n = 2x = 22$, the other *Nephelium* species are $x = 11$ (Seibert, 1992). According to the literature, it is assumed that $x = 11$ represents the haploid number; if so, the origin of the embryos within the ovule has not been determined. Neotropical fruit species include the akee and the Spanish lime, which are grown on a small commercial scale in countries in the Caribbean region.

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20.1 *Dimocarpus longan* Longan and *Litchi chinensis* Litchi

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1. Introduction

1.1. Botany and history

The litchi is considered to be a native of the region bounded by southern China, Vietnam and northern Malaysia (Menzel, 1992), and is widely grown as a commercial crop throughout the subtropics of the world. The longan probably originated in the mountains of Burma and southern China (Choo and Ketsa, 1992), and is distributed widely as a fruit crop in the subtropics. The litchi and longan are considered to be understorey and smaller canopy evergreen trees primarily of the tropical and subtropical Asian rainforest, and they rarely exceed 30 m height (Maity and Mitra, 1990; Menzel *et al.*, 1990). Both species generally have a dome-shaped canopy, although this can vary depending on the cultivar. The trees have similar morphology. The bark of longan is corky; leaves of both species are compound: 2-4(-6)-jugate and 2-4(-5)-jugate for longan and litchi, respectively. Flowers are borne on terminal panicle inflorescences. Flowering within a panicle consists of a sequence of opening of pistillate, hermaphroditic and staminate flowers. This ensures cross-pollination by insects. The fruit are drupes with a single seed enveloped within a fleshy, translucent edible aril. The seeds are highly

recalcitrant and lose viability within a few days. The exocarp of litchi is red, whereas that of longan is yellow-brown. There is a tendency to alternate bearing.

The litchi has been domesticated for approximately 3000 years, but has only spread beyond South-east Asia within recent historic time due to the recalcitrant nature of its seed and the exacting requirements for its cultivation (Maity and Mitra, 1990). *Litchi chinensis* belongs to the subfamily *Nepheleae* and is the only species within the genus *Litchi*. There are three subspecies within *L. chinensis*: ssp. *chinensis*, which is the common litchi; ssp. *philippinensis*, which is only known in the Philippines and is rarely cultivated; and ssp. *javensis*, which is found in west Java and southern Malaysia under cultivation in the humid lowlands.

The longan is one of seven species within the genus *Dimocarpus*, and is the only species in the genus that has economic importance. There are two subspecies within *Dimocarpus longan* ssp. *longan* and four varieties: (i) ssp. *longan* var. *longan*, the common longan from southern China to Myanmar; (ii) ssp. *longan* var. *longepetiolulatus* from southern Vietnam; (iii) ssp. *malesianus* var. *malesianus* from Malesia and Indochina; and (iv) ssp. *malesianus* var. *echinatus* from Borneo and the Philippines (Choo and Ketsa, 1992).

1.2. Importance

Production data for litchi and longan are not included in the FAOSTAT database. According to Menzel (1992), the leading litchi producing countries are China, Taiwan, India, Madagascar and Thailand, in that order. There is also significant production in South Africa, Australia and the USA. Leading export countries include Thailand, Taiwan and South Africa. Longan is grown commercially in Thailand, China, Taiwan, Australia and the USA.

1.3. Breeding and genetics

There has been little effort to develop improved longan and litchi cultivars using classical breeding procedures. Consequently, there is little genetic information available. According to Menzel (1992), the genetic diversity within litchi germplasm collections is limited, since most of the cultivars were selected for south China conditions. This conclusion has been confirmed by Ding *et al.* (2000), who observed limited DNA polymorphism among cultivated litchis. There have been no efforts to collect material from the original habitats. The same limitations for breeding are true also for longan. The chromosome number of longan is $2n = 2x = 30$ (Choo and Ketsa, 1992). The chromosome number of litchi is $2n = 2x = 28, 30$ or 32 (Menzel, 1992). Trees are very heterozygous, and are normally vegetatively propagated by air layering or grafting on to seedling rootstocks. There are no reported rootstock-related problems that limit commercial production of these crops.

1.3.1. Scions

Major breeding objectives

Shelf-life. Neither litchi nor longan has become a major tropical/subtropical crop because of the relatively brief harvest period and the rapid decline of fruit quality and appearance after harvesting. The fruit are non-climacteric, and unripe fruit are unable to ripen in storage. Litchi fruit, in particular, lose their bright red colour within 2–3 days after picking.

Diseases. Some of the important litchi cultivars, e.g. 'Tai So' (also known as 'Mauritius'), are very susceptible to anthracnose caused by *Colletotrichum gloeosporioides* Penz. Control measures in the humid subtropics currently include application of fungicide from the time of flowering until shortly before harvesting.

Yield. Litchi and longan trees are irregular bearers, and selections that have been made for a specific growing region often bear very erratically elsewhere. The short harvest period and irregular bearing make marketing very difficult, and prices for these fruit are consequently lower than would be expected, particularly at the peak of harvesting. High yields with regular bearing are essential in order to fully exploit the potential of local, national and international markets.

Fruit quality. In traditional growing areas, the most valued litchi and longan fruit have small aborted seeds, which are referred to as 'chicken tongues'. These fruit have a high flesh to seed ratio. This trait is apparently fairly frequent in some cultivars, e.g. 'Xia fanzhi', and in their progeny. Larger fruit size, particularly in longan, is desirable.

Breeding accomplishments. In the absence of breeding programmes, the vegetatively propagated selections of longan and litchi have all been derived from openly pollinated seedling populations. McConchie *et al.* (1994) reported that longan and litchi are partially sexually compatible, and intergeneric hybrids can be recovered, but only if the litchi is the maternal parent. Among the hybrid progeny, some of the plants produced seedless fruits, which could have interesting implications for selection of superior, seedless, interspecific fruit.

2. Molecular Genetics

2.1. Molecular markers

There have been several reports in which isozyme and random amplified polymor-

phic DNA (RAPD) markers have been utilized for the identification of litchi cultivars (Aradhya *et al.*, 1995; Degani *et al.*, 1995a; Ding *et al.*, 2000), for determining the pollen parent effect on fruitlet abscission (Degani *et al.*, 1995b) and for measuring the effect of pollen parent on outcrossing, yield and fruit characteristics (Stern *et al.*, 1993). Aradhya *et al.* (1995) demonstrated that a number of accessions and cultivars have been misnamed based upon differences or similarities in enzyme polymorphisms. Although Degani *et al.* (1995a) considered that isozyme polymorphism in litchi is fairly wide and could be useful for taxonomic and systematic purposes, Ding *et al.* (2000), working with RAPDs, considered that DNA polymorphism was less than expected.

3. Micropropagation

It has not been possible to stimulate the *in vitro* proliferation of axillary shoots of elite selections of either longan or litchi. The initiation of multiple shoot proliferation of litchi from shoot apices of *in vitro*-germinating seeds (i.e. non-clonal) has been reported by Kantharajah *et al.* (1992) and Das *et al.* (1999). In addition, Das *et al.* (1999) described the initiation of multiple shoot proliferation from *in vitro*-grown litchi seedlings. Multiple shoot formation occurred from the cotyledonary nodes following exposure to 88.8 μ M benzyladenine (BA) in liquid Murashige and Skoog (1962) (MS) medium with sterile filter paper bridges and from 4- to 5-week-old *in vitro*-grown seedlings that were exposed to 100 μ M BA on alternate days (Das *et al.*, 1999). Rooting of individual shoots was induced by pulsing the shoots with 122.6 μ M indolebutyric acid (IBA) for 15 min followed by root development in potting mixture.

Zamora *et al.* (1988) described the initiation of multiple shoots of rambutan from shoot tip and nodal cultures derived from *in vitro*-germinated seedlings. Induction of root development from individual shoots from these cultures was possible.

4. Somatic Cell Genetics

4.1. Regeneration

A prerequisite for applying many biotechnology tools to perennial tree species is the availability of a reliable procedure for regenerating elite tree selections (cultivars) from cell cultures. Embryogenic cultures of litchi and longan have been induced; however, the studies involving litchi have not utilized elite material, although embryogenic longan cultures have been induced from mature-phase plants. Desai *et al.* (1986) described the regeneration of soapnut (*Sapindus trifoliata* L.) by somatic embryogenesis from leaves of elite trees. The response of soapnut embryogenic cultures to sodium chloride was measured by Unnikrishnan *et al.* (1991). They determined that somatic embryos could tolerate up to 200 ml/m³ sodium chloride, and that lower concentrations stimulated proliferation of embryogenic cultures.

4.1.1. Somatic embryogenesis

Longan. There have been several studies that have focused on the induction of embryogenic cultures from non-elite, i.e. seed-originating, longan tissues (Wei and Yang, 1981; Lai *et al.*, 1995, 1997a,b, 1998, 2000; Lai and Chen, 1998). More significantly, embryogenic longan cultures have been induced from 30-year-old 'Kohala' and 'Selection 12' trees (Litz, 1988; S. Raharjo and R.E. Litz, unpublished data). The explanted tissue consists of entire surface-sterilized leaflets of compound leaves in recent vegetative flushes. Since the original report, the induction medium has been somewhat modified, and consists of B5 (Gamborg *et al.*, 1968) major salts (without (NH₄)₂SO₄), MS minor salts and organic components, 400 mg/l glutamine, 60 g/l sucrose, 2.3–9.3 μ M kinetin, 2.3–4.5 μ M 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.0 g/l gellan gum. Tissue cultures are incubated in darkness at room temperature (25°C). Embryogenic cultures first appear approx. 3–4 weeks after explanting. Embryogenic cultures consist almost entirely of proembryonal cells and masses (PEMs). On induction medium,

globular and early stage cotyledonary somatic embryos develop from the embryogenic cultures distal to the surface of the plant growth medium. For induction, maintenance on semi-solid medium, maturation and germination, cultures can be grown in standard Petri dishes.

For efficient maintenance of embryogenic longan cultures, it is essential to inoculate embryogenic cultures into liquid medium (Lai *et al.*, 1995). Usually 300 mg embryogenic culture is inoculated into 80 ml maintenance medium in a 250 ml Erlenmeyer flask: B5 major salts, MS minor salts and organic components, 400 mg/l glutamine, 60 g/l sucrose and 4.5 μ M 2,4-D. Suspension cultures are maintained at approx. 100 rpm on a rotary shaker in semi-darkness at room temperature, and subcultured into fresh medium of the same formulation at 2–3-week intervals. Proliferation of embryogenic cultures is by the induction of embryogenic cells and secondary embryos from the protoderm of PEMs.

Development and maturation can be initiated from embryogenic cultures by subculture on to semi-solid maturation medium. Maturation medium consists of B5 major salts, MS minor salts and organic components, 400 mg/l glutamine, 20 g/l sucrose, 10% (v/v) filter sterilized coconut water and 2.0 g/l gellan gum. Somatic embryos are able to develop to maturity on this medium. Following the initiation of germination, cultures are routinely transferred to light (60 μ mol/m²/s) with a 16 h photoperiod supplied by cool white fluorescent bulbs. Germination occurs approx. 10–14 days following transfer to light conditions, and precedes shoot formation by a few days. Germinated plantlets can be transferred to potting soil and hardened under intermittent mist with minimal losses.

Litchi. Embryogenic litchi cultures have been induced only from non-elite, i.e. zygotic, embryos of 'Xiafanzhi' (Zhou *et al.*, 1996; Yu and Chen, 1997; Yu *et al.*, 2000) and 'Brewster' (also known as 'Chen Zi') (A. Celo and R.E. Litz, 1996, Florida, unpublished data). Immature fruit, approx. 3–4 weeks after flowering, are surface-sterilized

and dissected under aseptic conditions in order to remove the zygotic embryo for use as an explant. Induction medium has varied slightly between the two groups indicated above. In Florida, USA, the induction medium described for elite longan has been used effectively. Cultures are maintained in standard size Petri dishes in darkness at room temperature. Embryogenic cultures generally appear after 5–6 weeks. Cultures consisted of somatic embryos of different development stages: from globular to cotyledonary (Witjaksono and R.E. Litz, Florida, 1999, unpublished data). On the other hand, Yu and Chen (1997) described the induction of friable embryogenic masses. These differences in induction are probably genotype-dependent.

It is possible to maintain embryogenic litchi cultures either on semi-solid medium or as suspensions (Yu and Chen, 1998). Maintenance medium consists of induction formulation. Embryogenic cultures proliferate by the formation of secondary somatic embryos, many of which continue to develop in the presence of 2,4-D to advanced cotyledonary stage, and some friable PEMs. Many of the somatic embryos are hyperhydric. Friable PEMs can be maintained by subculture on semi-solid medium. Yu and Chen (1997) reported that maintenance of embryogenic cultures on semi-solid medium resulted in a mixture of PEMs and developing somatic embryos. In order to inhibit somatic embryo development, they incorporated 29.4 μ M silver thiosulphate in the medium, and obtained friable cultures consisting of PEMs. Approximately 1.5 g friable PEMs is inoculated into 30 ml of liquid induction medium in 125 ml Erlenmeyer flasks and diluted three times at weekly intervals. Maintenance of PEMs in suspension for up to a year is accomplished by alternating liquid with semi-solid (containing silver thiosulphate) media. Cultures are maintained on a rotary shaker at 100 rpm in darkness or semi-darkness at room temperature.

Maturation of embryogenic cultures can be initiated by their subculture on to maturation medium and incubation in darkness; however, germination and conversion are

poor. Yu *et al.* (2000) observed improved rates of germination of somatic embryos from embryogenic protoplasts (see Section 4.1.3).

4.1.2. Haploid recovery

Longan. Induction of embryogenic cultures from microspores of cultured longan anthers has been reported (Yang and Wei, 1984; Wei, 1990). Some success has been achieved with the following cultivars: 'Dong Bi', 'Hong He Zi', 'Oolong Mountain' and 'You Tan Ben'. Unopened staminate and hermaphroditic flowers are collected from longan panicles. Closed flower buds containing mid- to late-uninucleate stage are pre-treated at 4°C for 24 h. Following surface sterilization and washing, the buds are dissected under aseptic conditions, and the anthers are removed and cultured on semi-solid induction medium: MS supplemented with 4.6 μ M kinetin, 9.0 μ M 2,4-D, 5 g/l activated charcoal and 50 g/l sucrose. Anther cultures are maintained at 25–27°C in darkness. Following induction, the embryogenic (haploid) cultures are transferred on to semi-solid maturation medium containing 2.2–4.4 μ M BA, 0.5 μ M naphthaleneacetic acid (NAA) and 16 g/l sucrose. Germination occurs on semi-solid Anderson's rhododendron basal medium (Anderson, 1975) supplemented with 1.3 μ M BA, 0.6 μ M indole-3-acetic acid (IAA) and 16 g/l sucrose. Conditions for haploid embryo development and germination included a 12 h photoperiod at 80 μ mol/m²/s. Plantlets derived from microspores are slow-growing. The chromosome number of cells in root tips of regenerated plants was reported to be $n = x = 15$.

Litchi. Unopened staminate and hermaphrodite 'Chenzhi' and 'Gushan Jiaohé' flowers approx. 3–4 mm length are utilized as a source of anthers with microspores at the optimum late uninucleate stage of development (Fu and Tang, 1983; Fu, 1990). The flower buds are surface-sterilized, washed with sterile distilled water and dissected under aseptic conditions. Anthers are removed and plated on induction medium consisting of MS salts and organic compo-

nents, 4.5–9.0 μ M 2,4-D, 4.6–9.3 μ M kinetin, 2.7–5.4 μ M NAA and 16 g/l sucrose. Anther cultures are incubated at 25°C with a 10 h photoperiod (60 μ mol/m²/s). Approximately 10 weeks after explanting, embryogenic cultures are apparent. Embryogenic (haploid) cultures can be maintained on induction medium. In order to initiate haploid embryo development and maturation, embryogenic cultures have been subcultured on MS supplemented with 2.3 μ M kinetin, 0.5 μ M NAA, 500 mg/l casein hydrolysate, 400 mg/l royal jelly and 16 g/l sucrose. Early heart stage embryos are transferred to MS with 2.3 μ M kinetin, 2.9 μ M gibberellic acid (GA₃), 500 mg/l casein hydrolysate, 400 mg/l royal jelly, 1.7 g/l glutamine and 16 g/l sucrose; on this medium, the embryos develop to maturity and germinate. Examination of chromosomes in cells of root tips of regenerated plants indicated that most of the cells were haploid ($n = x = 15$), although aneuploid and triploid cells were also observed.

4.1.3. Protoplast isolation and culture

Longan. Lai (1997) and Lai and Chen (1996) reported the isolation and culture of protoplasts from embryogenic longan suspension cultures. The cultures were derived from non-elite material, i.e. zygotic embryos. Embryogenic suspension cultures were incubated in an enzyme mixture consisting of 1% Onozuka R-10 cellulase and 1% pectinase with 13% (w/v) mannitol for 14 h. After washing the protoplasts, they were suspended in calcium alginate beads. Heart stage somatic embryos that developed from protoplasts were transferred to maturation medium.

Litchi. Yu *et al.* (2000) described the isolation and culture of protoplasts from embryogenic suspensions of litchi. Four-day-old suspensions were collected by low-speed centrifugation and plasmolysed in CPW (Frearson *et al.* (1973) as modified by Grosser and Gmitter (1990)) that was supplemented with mannitol (13% w/v) for 1 h. The cultures were resuspended in filter-sterilized enzyme solution containing 0.8% (w/v) cellulase

'Onozuka' RS, 0.4% (w/v) macerozyme R-10, 11% (w/v) mannitol and CPW salts. Following overnight incubation at 45 rpm in darkness at 26°C, the cultures were passed through sterile nylon fabric (37 μ m) and the mixture was centrifuged at 100 g for 5 min. The protoplasts were washed with CPW salts supplemented with 11% (w/v) mannitol, washed once with CPW without calcium and resuspended in 10% (v/v) mannitol containing 1.8% (w/v) sodium alginate. The protoplasts in suspension were added dropwise into sterile 1% (w/v) $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ in CPW solution. After 1 h the solution was replaced with MS medium supplemented with 2.3 μ M zeatin, 2% (v/v) coconut water, 0.45 M glucose and 0.1 M sucrose. Enhanced survival was obtained when a nurse culture consisting of embryogenic cultures embedded in calcium alginate was co-cultured with the encapsulated protoplasts. PEMs and globular somatic embryos were released and somatic embryo development was initiated on MS medium supplemented with B5 vitamins, 4.7 μ M kinetin, 0.5 μ M NAA, 500 mg/l glutamine, 8% (w/v) sucrose and 15 g/l agar in darkness.

In order to stimulate maturation and germination, somatic embryos were transferred to maturation medium consisting of MS major and minor salts, B5 vitamins, 500 mg/l glutamine, 5% (v/v) coconut water, 50 g/l sucrose and 9 g/l agar. For germination, mature somatic embryos were subcultured on to medium consisting of MS major and minor salts, B5 vitamins, 500 mg/l glutamine, 5% (v/v) coconut water, 30 g/l sucrose, 4.7 μ M kinetin, 14.4 μ M GA_3 and 7.0 g/l agar. Cultures were maintained under a 16 h photoperiod (80 μ mol/m²/s). A few plants have survived transfer to soil.

4.2. Genetic manipulation

4.2.1. Genetic transformation

Longan. Zheng *et al.* (2001) reported the transformation of somatic embryos derived from embryogenic cultures of zygotic embryos of 'Sieryuelongyan'. Somatic embryos were infected with different wild

strains of *Agrobacterium rhizogenes*, of which strain R1601 was most effective. The hairy roots were excised and cultured on semi-solid MS medium with 22.19 μ M BA and 23.23 μ M kinetin in order to stimulate secondary somatic embryo development. Plants were recovered from the transformed somatic embryos that had hairy roots, and transformation was confirmed by Southern hybridization and polymerase chain reaction (PCR).

Litchi. Litchi has been genetically transformed with two genes that are associated with resistance to pathogens (Witjaksono and R.E. Litz, Florida, 1999, unpublished data). Embryogenic cultures derived from zygotic embryos of openly pollinated 'Brewster' litchi were incubated for 3 days in liquid maintenance medium at 100 rpm with acetosyringone-activated *Agrobacterium tumefaciens* strain LBA4404. *A. tumefaciens* was electroporated with three different gene constructs, i.e. (i) pBI121 that contains the selectable marker *nptII* and the scorable marker *uidA*; (ii) pGPTV-BAR, in which chitinase and β -1,6-glucanase genes were inserted in a tandem order (pGPTV-BAR-CG); and (iii) the antifungal protein gene (pGPTV-BAR-AFP). The *nptII* and *uidA* genes in pBI121 are driven by the 35S promoter. The chitinase, β -1,6-glucanase and antifungal protein genes and *uidA* in pGPTV-BAR are driven by double 35S promoter. *A. tumefaciens* was then eliminated by incubating the cultures in maintenance medium supplemented with 200 mg/l cefotaxime for 2 weeks. Following the removal of *A. tumefaciens*, the embryogenic cultures were transferred on to semi-solid maintenance medium supplemented with 2 g/l phosphinothricin to select for pGPTV-BAR and 100 mg/l kanamycin to select for pBI121. Expression of the *uidA* gene by the X-Gluc histochemical reaction (Jefferson, 1987) 2 weeks after the end of selection showed differential transformation efficiency among the constructs. Transformation with pBI121 was most efficient, whereas pGPTV-BAR with chitinase and β -1,6-glucanase genes was least efficient.

4.2.2. Somatic hybridization

Preliminary studies have reported the fusion of protoplasts of litchi and longan (Lai and Chen, 1997). Although limited division of somatic hybrid cells was described, the recovery of somatic embryos has not been reported. The utility of litchi + longan somatic hybrids is uncertain due to their polyploidy and consequent problems of introgression of useful genes into either parent. Moreover, McConchie *et al.* (1994) reported that litchi and longan can be sexually hybridized, and intergeneric hybrids can be recovered.

4.3. Cryopreservation

The ability to store embryogenic longan and litchi cultures for extended periods is critical for many cell manipulation studies, because embryogenic cultures lose their morphogenic competence over time. Preliminary studies have demonstrated that embryogenic longan cultures can be cryopreserved in two ways: (i) stepwise cooling ($-1^{\circ}\text{C}/\text{min}$) to -80°C using 'Mr. Frosty'®, followed by plunging of cryovials into liquid nitrogen (-196°C); and (ii) by vitrification using the plant vitrification solution 2 (PVS2) formula (Sakai *et al.*, 1991), consisting of glycerol (30%), ethylene glycol (15%) and dimethyl sulphoxide (DMSO) (15%) for 15 min, followed by plunging the cryovials into liquid nitrogen (Matsumoto *et al.*, 2004). The standardization of this protocol should have an important impact not only for storage of experimental materials, but because the technology could be used as a backup for germplasm collections.

5. Conclusions

The basic *in vitro* protocol for manipulating elite longan selections is available, and could be utilized to address various plant breeding objectives using biotechnology procedures.

Comparable studies are necessary in order to define a *de novo* regeneration pathway for litchi from elite selections. Genetic transformation of litchi and certainly longan is feasible. Longan, because it can be regenerated from embryogenic cultures of elite selections with a high rate of conversion, could be genetically transformed with genes that encode for resistance to fungal disease, e.g. anthracnose, caused by *C. gloeosporioides* Penz. It should also be possible to apply *in vitro* mutation induction and selection procedures to address certain fungal disease problems of known longan cultivars. Genetic transformation could be utilized to develop the preferred 'chicken tongue' seeds using the *picata* gene from *Arabidopsis thaliana* that mediates seedlessness. Protoplast technology could be harnessed to produce somatic hybrids between haploid and diploid longans and between haploid and diploid litchis as a way of producing seedless triploids. The resolution of problems associated with rapid deterioration of fruit postharvest are more difficult to address at this time since the fruit of neither longan nor litchi is climacteric.

This review of subtropical fruit species within the *Sapindaceae* should provoke interest in the potential for applying biotechnology for improving not only longan and litchi, but also the other species of this family, particularly rambutan and the related *Nephelium* species. The *de novo* regeneration of elite selections of litchi and rambutan has not been reported. This is a prerequisite for applying somatic cell genetic approaches to elite selections of these species. Following the successful regeneration of longan and soapnut from embryogenic culture derived from mature trees, comparable studies should be undertaken with litchi and rambutan.

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Sterculiaceae

The former family *Sterculiaceae* included > 50 genera (Purseglove, 1968) and approx. 1000 species (Alverson *et al.*, 1998, 1999), mostly pantropical small trees and shrubs. The *Sterculiaceae* taxa are not easily described by unique or any combination(s) of characters (Whitlock *et al.*, 2001). Recent phylogenetic studies involving several genera from the order *Malvales* concluded that the *Sterculiaceae*, as well as the families *Tilliaceae* and *Bombacaceae*, are not monophyletic, and should be combined to form a broadly defined *Malvaceae sensu lato* (s.l.) (Judd and Manchester, 1997; Alverson *et al.*, 1999; Bayer *et al.*, 1999). This reclassification of the *Malvaceae* has been supported by molecular phylogenetic analyses using sequences of the plastid genes *rbcL* (Alverson *et al.*, 1998; Bayer *et al.*, 1999), *ndhF* (Alverson *et al.*, 1999; Whitlock *et al.*, 2001) and *atpB* (Bayer *et al.*, 1999). According to the new classification, the species from the former *Sterculiaceae* were reclassified into subfamilies *Byttnerioideae*, *Helicterioideae*, *Dombeyoideae* and

Sterculioideae of the family *Malvaceae s.l.* (Bayer *et al.*, 1999).

Within the *Malvaceae s.l.*, *Theobroma cacao* (cacao) is the only fruit tree species of major economic importance. *Theobroma grandiflorum* and *Theobroma bicolor* are cultivated on a small scale for extraction of the seed-surrounding pulp. *T. grandiflorum* (cupuassu) is increasing in importance in Brazil and elsewhere (Velho *et al.*, 1990), with at least 20,000 ha under cultivation in the Amazonian states of Brazil (Ribeiro, 1997). Other species of the former *Sterculiaceae* include locally important crops, e.g. various *Cola* spp. (*C. acuminata*, *C. nitida*, *C. anomala*, *C. verticillata*), which produce caffeine-rich edible seeds for beverages or for direct chewing, mostly in West Africa (Purseglove, 1968). *Sterculia urens* is used for production of gum karaya from bark incisions mainly in India and is used as a substitute for gum tragacanth; the species has become an endangered species (Purohit and Dave, 1996; Sunnichan *et al.*, 1998).

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21.1 *Theobroma cacao* Cacao

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1. Introduction

1.1. Botany and history

The genus *Theobroma* is of exclusively neotropical origin, consisting of small understorey trees of the lowland rainforest, and distributed from the Amazon basin through southern Mexico (Purseglove, 1968). Cuatrecasas (1964) recognized 22 *Theobroma* species and, based on mode of germination, branching pattern and flower morphology, subdivided the genus into six sections: *Andropetalum* (*T. mammosum*); *Glossopetalum* (*T. angustifolium*, *T. canumanense*, *T. chocoense*, *T. cirmolinae*, *T. grandiflorum*, *T. hylaeum*, *T. nemorale*, *T. obovatum*, *T. simiarum*, *T. sinuosum*, *T. stipulatum* and *T. subincanum*); *Oreanthes* (*T. bernouillii*, *T. glaucum*, *T. speciosum*, *T. sylvestre* and *T. velutinum*); *Rhytidocarpus* (*T. bicolor*); *Telmatocarpus* (*T. gileri* and *T. microcarpum*); and *Theobroma* (*T. cacao*). With the exception of the cultivated species (*T. cacao*, *T. bicolor* and *T. grandiflorum*), most species have a restricted geographical distribution, with major separation between species occurring on the eastern side of the Andes (Amazonia) and on the Pacific side (Colombia, Ecuador and Central America) (Baker *et al.*, 1953). All sections have representatives on both sides of the Andes, except for *Andropetalum* (*T. mammosum*), which occurs only in Costa Rica.

The genus chromosome number is $2n = 2x = 20$ (Muñoz, 1948). Phylogenetic relationships of *Theobroma* were initially based on comparative morphology (Cuatrecasas, 1964). The classification of *Theobroma* species into sections was supported by using fatty acid, sterol, tocopherol and tocotrienol composition in seed fat as chemotaxonomic characters (Carpenter *et al.*, 1994). Fatty acid and triacylglycerol composition of seed fat and total seed protein profiles on polyacrylamide gel electrophoresis could also clearly separate sections according to the traditional classification (Gilbert-Escrivá *et al.*, 2002; Silva *et al.*, 2002). The subdivision of *Theobroma* into sections has also been supported by ribosomal DNA (rDNA) polymorphism (Figueira *et al.*, 1994). A partial survey of purine alkaloids in seeds detected the presence of caffeine and theobromine only in *T. cacao*, while all other *Theobroma* species, except for *T. obovatum*, have tetramethylurate (theacrine) (Hammerstone *et al.*, 1994). Although most sections of *Theobroma* have distinct characters, the relationships among them are unclear (Whitlock and Baum, 1999). Based on parsimony analysis of vicilin gene sequence, *Herrania* and *Theobroma* are considered monophyletic genera, but the monophyly of *Theobroma* is only weakly supported (Whitlock and Baum, 1999). All *Theobroma* sections appear to be monophyletic, except for *Glossopetalum*. Identical results were

obtained using sequence data of the 21 kDa trypsin-inhibitor gene (Silva, 2000).

Cacao was domesticated in pre-Columbian times in Meso-America, probably by the Olmecs and/or Mayas (Coe and Coe, 1996), who used the seeds to prepare a highly prized beverage once restricted to royalty (Bergmann, 1969) and so valued that seeds were used as currency (Paradis, 1979). The words cacao and chocolate are derived from the Nahuatl *cacahuatl* (cacao water) and *chocolatl* (hot water) (Coe and Coe, 1996). The substitution of maize meal and *Capsicum* pepper from the original Meso-American chocolate recipe by sugar and vanilla made the beverage more palatable to Europeans. This stimulated exports from Mexico, Guatemala and El Salvador. Despite high prices, the beverage became popular during the 17th century, and production was extended to non-traditional areas, e.g. Venezuela, Trinidad and Jamaica (Purseglove, 1968). Venezuela became the major exporter, and Ecuador became the major producer of the fine flavoured cacao 'Arriba' from the local type called *cacao nacional* (Wood and Lass, 1987). The development of a cocoa mass defatting process and the invention of the milk chocolate bar during the 19th century, associated with lower import duties, turned chocolate into a more accessible product with an increasing demand (Coe and Coe, 1996). Production was introduced into Ghana in 1879 and, during the first half of the 20th century, the bulk of cacao originated in West Africa, mainly Ghana and Nigeria, and from Brazil. More recently, changes in production have occurred, with increased production in Ivory Coast and Indonesia, whereas production in Brazil and Malaysia has declined.

The traditional classification of cacao assumes three major morphogeographic groups: Criollo, Forastero and Trinitario. Criollos and Forasteros have distinct historical and commercial features (Cheesman, 1944). This classification system, based on pod and seed characters, is at best imprecise. Domesticated in Meso-America, Criollos are characterized by pods that are red or yellow when ripe, deeply furrowed, warty, with a pointed end and a thin husk, containing

plump seeds with white or pale violet cotyledons, and with a superior flavour. Historically, all non-Criollo cacaos were considered to be Forasteros, but this term is currently used to describe cacao types that have non-pigmented pods and a thick and hard pod husk, with flat, dark purple seeds. The Forasteros can be subdivided into Upper Amazonian (wild or semi-wild cacaos as described by Pound (1938)) and Lower Amazonian, characterized by a rather uniform pod type called amelonado (green pod with smooth, shallow furrows, melon-shaped with a blunt end and slight bottle-neck). Lower Amazonian cacaos, which now constitute the most prevalent cultivated type worldwide, grow wild in the Guyanas and in the eastern region of the Amazon, and were probably partially domesticated by pre-Colombian peoples for the aromatic pulp (Barrau, 1979). Trinitarios are considered to be either an intermediate type between Criollo and Forastero (Lockwood and Gyamfi, 1979) or a hybrid, which displays characteristics that include the total range of variation (Cheesman, 1944). Trinitarios have not been found in the wild, and are presumed to derive either from hybrid populations (Venezuelan Criollo $\times$ Lower Amazonian Forastero) in the Orinoco River estuary, or from a cross between a Forastero introduction in Trinidad and Criollo plants that survived the destruction of the industry in 1727 (Cheesman, 1944). Because Criollos and Forasteros are so heterogeneous, the resulting hybrids might not be distinct from the parental populations, making Trinitarios impossible to define except by origin.

There are currently two hypotheses regarding the origin and distribution of *T. cacao*. Based on the occurrence of spontaneous cacao plants displaying the whole intraspecific variation for pod morphology in the upper Amazonian region, Cheesman (1944) proposed that the area between the Caquetá, Napo and Putumayo rivers is the centre of diversity for the species. Cacao might have been transported across the Andes by humans and possibly by sea (Wolters, 1999), accounting for populations in Central and northern South America. The geographical separation caused by the Andes

and the Panama isthmus might have caused the differentiation of cacao into the two morphogeographic groups (Criollo and Forastero), but not sufficiently to isolate both groups into two subspecies (Cheesman, 1944). The largest diversity of cacao from the Upper Amazonian region has been confirmed by evaluation of field resistance to *Phytophthora* pod rot (Pires *et al.*, 1994) and to *Crinipellis perniciosa*, causal agent of witches' broom disease (Pires *et al.*, 2000b); by isozymes (Lanaud, 1987a; Ronning and Schnell, 1994; Warren, 1994); by restriction fragment length polymorphisms (RFLPs) using rDNA gene (Laurent *et al.*, 1993a; Figueira *et al.*, 1994) and complementary DNA (cDNA) probes (Laurent *et al.*, 1994a,b; N'Goran *et al.*, 1994); random amplified polymorphic DNAs (RAPDs) (Figueira *et al.*, 1994; N'Goran *et al.*, 1994; Whitkus *et al.*, 1998); and microsatellites (Lanaud *et al.*, 1999a; Motamayor and Lanaud, 2001). Lerceteau *et al.* (1997) detected a larger allelic diversity for genotypes from Peru and Ecuador.

Alternatively, Cuatrecasas (1964) postulated that a natural cacao population once extended from the Guyana-Amazon region through southern Mexico, and ultimately developed into two different forms. One of the forms was the subspecies *T. cacao* subsp. *cacao* from Central America and Mexico, and its cultivated form represented the Criollo morphogeographic group, actively selected by Meso-American peoples (Cuatrecasas, 1964). The other form was *T. cacao* subsp. *sphaerocarpum* from South America, representing the Forastero group. Evidence for this hypothesis includes the discovery of wild *T. cacao* subsp. *cacao* in the Lacandona forest in Chiapas, Mexico (Cuatrecasas, 1964), and the recent finding of cacao trees at 'cenotes' (sacred sink holes) in Yucatan, Mexico (Gómez-Pompa *et al.*, 1990). Laurent *et al.* (1993b) analysed 177 cacao genotypes using heterologous cytoplasmic probes and detected more variability in Criollo genotypes than in Forasteros. Classification of genotypes into Criollo and Forastero by isozyme analysis (Ronning and Schnell, 1994; Warren, 1994), by RFLP using rDNA (Laurent *et al.*, 1993a) or cDNA probes (Laurent *et al.*, 1994b; N'Goran *et al.*, 1994)

and RAPDs (N'Goran *et al.*, 1994), provides additional evidence for Cuatrecasas's hypothesis, although separation of genotypes into Criollo and Forastero might derive from founder effect, selection or genetic drift by isolation and does not necessarily represent origin differences. 'Wild' cacao from *cenotes* in Mexico was evaluated based on 105 RAPD loci amplified by 24 arbitrary primers, and compared with cultivated Criollo, Forastero and wild South American genotypes (de la Cruz *et al.*, 1995). The Yucatan 'wild' genotypes were genetically distinct from the other cacao, and the cultivated Criollos were more similar to wild South American genotypes. However, the occurrence of exotic plants at the *cenotes*, such as *Musa*, *Citrus* and *Cocos* (Gómez-Pompa *et al.*, 1990), did not exclude the possibility of cacao introduction from other areas, including Forasteros. In a subsequent study, Whitkus *et al.* (1998) evaluated the genetic diversity between individuals collected at Yucatan, Mexico, with other putative wild cacao from Chiapas, Mexico, and cultivated Criollos from Chiapas and Tabasco, Mexico, wild and cultivated Forastero genotypes and Trinitarios, based on 57 informative RAPD loci amplified by 13 primers. The cultivated populations from Chiapas and Yucatan formed a significantly different group from cultivated and wild Forastero genotypes from South America, while plants considered as Criollo were less similar to individuals from Chiapas and Yucatan than to wild and cultivated Forastero genotypes.

Motamayor *et al.* (2002) analysed cacao genotypes considered to be ancient Criollos, collected in Venezuela, Colombia, Guatemala, Nicaragua and Mexico (including samples from the Lacandona forest and archaeological sites). Based on the 66 RFLP alleles derived from 17 cDNA and eight genomic probes and 150 microsatellite alleles amplified from 16 loci, it was concluded that the ancient Criollo genotypes from South and Central America are identical, with low genetic diversity and high homozygosity. The putative ancient Criollos are more similar to individuals from Colombia and Ecuador, than those from Colombia and

Ecuador are to the Peruvian ones (Motamayor *et al.*, 2002). The modern cultivated Criollos in germplasm collections have Forastero introgression, and genetically resemble genotypes considered Trinitarios, with elevated diversity (Motamayor *et al.*, 2002). These results suggest that the Criollo group might in fact have originated from a few individuals from South America that were spread to Central America by humans (Lanaud *et al.*, 1999a, 2001; Motamayor and Lanaud, 2001; Motamayor *et al.*, 2002).

1.2. Importance

Its fat-rich seeds are the unique source of cocoa solids and cocoa butter, raw materials for the chocolate, confectionery and cosmetic industries. The worldwide confectionery industry was estimated to be worth c. US\$150 billion in 2001, producing 12.8 Mt, half being chocolate alone (ICCO, 2002). Cacao is grown in more than 50 countries (Knight, 2000) by > 2 million growers (Ruf, 1992). Cacao world production may reach 2.807 Mt in 2001/02, while grindings are forecast to attain 2.859 Mt, a deficit covered by current available stocks of 1.077 Mt (ICCO, 2002).

1.3. Breeding and genetics

Cacao breeding programmes have been based on a narrow genetic base and restricted genetic information (Kennedy *et al.*, 1987), and have lacked continuity because of price and political instability. Therefore, genetic improvement has not had a major impact on the cacao industry, except in Cameroon, Ivory Coast and Malaysia, where the cacao industry was established fairly recently (Kennedy *et al.*, 1987). Brazil and Ecuador are also benefiting from renewing traditional stands with selected witches' broom-resistant planting material (Pinto and Pires, 1998).

Each major cacao-producing region is affected by a specific devastating disease (Figueira and Janick, 1995). Witches' broom disease, caused by *C. perniciosa*, occurs only in South America and the Caribbean region.

Monilia pod rot, caused by *Moniliophthora roreri*, is a problem in South and Central America. Cacao swollen shoot badnavirus (CSSV) occurs in West Africa, and vascular streak dieback, caused by *Oncobasidium theobromae*, is limited to Malaysia, Indonesia and Papua New Guinea. The most important pathogen is the pantropical ubiquitous *Phytophthora* complex (*P. palmivora*, *P. capsicii*, *P. citrophthora*, *P. megakarya*), causing black-pod rot in all regions. The most virulent species (*P. megakarya*) is limited to certain parts of West Africa. Cacao is not affected by any serious bacterial diseases. Pests are of less importance in cacao production, except for cacao pod borer (*Conopomorpha cramerella*), which only occurs in South-east Asia. In West Africa, mirids are important pests which, together with mealybugs, are responsible for the transmission of CSSV. Various mirid species (*Sahlbergella singularis*, *Distantiella theobromae* and *Helopeltis* spp.) deposit eggs on young stems, branches and pods, that are later eaten by the larvae.

Cacao breeding was initiated in the 1920s with phenotypic selection of local populations in most of the cacao-producing countries (Trinidad, Indonesia, Ghana, Nigeria, Costa Rica and Brazil) for improved yields, while maintaining commercial quality (Toxopeus, 1969). Selected plants were intended for either clonal distribution or after evaluation of self-pollinated progenies to be distributed as clonal seed. Little improvement was obtained either by selfing or by crossing local selections from homogeneous populations (mainly West Africa and Brazil).

The first case of heterosis in cacao was observed when introduced Trinitarios in West Africa were crossed and compared with selfed progenies from local selections (Russel, 1952). Until then, exchange and collection of germplasm were mostly concerned with quality improvement. The outbreak of witches' broom in Trinidad in the early 1930s led to the search for genetic resistance. Resistance was not found within the Trinidad population, and expeditions were organized by Pound (1938, 1942) to collect germplasm in the Upper Amazonian region and from Ecuadorian farms, where witches'

broom had appeared a few years earlier (Toxopeus, 1969). Resistance to witches' broom was found in two genotypes collected near the Ucayali River in Peru, i.e. the Scavina clones ('Sca 6' and 'Sca 12'), and in other material. In West Africa, resistance to CSSV was not found in local populations (Adu-Ampomah *et al.*, 1996). Crosses among Upper Amazonian accessions were introduced into Ghana (Toxopeus, 1964); heterosis was observed in the progenies from these crosses. An F_1 population was selected for vigour, precocity, yield and flavour, and open-pollinated pods from superior selections were used to establish seed gardens (Toxopeus, 1964). Seeds from these gardens (named 'F₃ Amazon') were distributed to farmers and, although the material was not resistant to virus and mirids, it recovered quickly after attack and tolerated marginal production areas. This 'variety' can be considered as a semi-synthetic or a panmictic population, because parents were neither rigorously selected nor tested before the population synthesis (Lockwood, 1985).

Interpopulation heterosis was demonstrated for crosses between Upper Amazonian Forastero and Trinitario, Criollo or an unrelated Forastero, and represents the basis for modern cacao breeding (Kennedy *et al.*, 1987). Selected Upper Amazonian parents are usually crossed with local selections and/or a Trinitario selection, in order to keep traditional quality, yield and resistance to current pests and diseases. Self-incompatibility in the Upper Amazonian genotypes has been explored in order to avoid hand pollination for hybrid seed production. Currently, only a small number of Upper Amazonian parents have been evaluated and used in improvement programmes, restricting the genetic base of modern cacao. Further progress might be expected from a wider evaluation of various parents. Mixtures of F_1 hybrids are used to avoid sterility and susceptibility to disease and pest outbreaks; however, the restricted number of hybrids has caused cross-incompatibility problems in some hybrid combinations, limiting production.

The initial heterotic vegetative vigour from crosses between highly heterozygous

parents, which appear superficially as uniform progeny, masks intense segregation (Warren and Kennedy, 1991). Disparities between predicted performance (including disease resistance) of cacao hybrids and actual yields under field conditions have inhibited the acceptance of this material by growers, and have underscored the lack of basic genetic knowledge (Hunter, 1990). Variable yield performance of cacao hybrids in commercial production has been observed, where approx. 20% of trees produce 80% of the crop (Hunter, 1990). To overcome the natural variation occurring in hybrid progenies, two approaches have been used to produce more homogeneous parents: (i) inbreeding for a few generations (Bartley, 1967); and (ii) production of double-haploids originating from spontaneous haploid seedlings (Dublin, 1978; Lanaud, 1987a). However, selfing takes a long time, and spontaneous haploids occur at low frequency (10^{-4} to 10^{-3}), depending upon genotype (Dublin, 1972, 1978), and are characterized by decreased fertility and vigour (Lanaud, 1987b; Lanaud *et al.*, 1988).

Long-term genetic improvement of cacao populations based on recurrent selection methods is a promising alternative for short-term exploitation of heterosis, favouring the increase in frequency of favourable alleles to keep continuous genetic gains over generations (Lockwood and Pang, 1993). The limited options for exploitation of a complex combination of traits of interest in hybrids, plus the increased genetic base, favour the use of population breeding methods (Eskes *et al.*, 1993). Yield might have an important additive genetic component (Lockwood and Pang, 1993). The long juvenile period is a major constraint. Various types of planting material can be produced using this approach (Lockwood and Pang, 1993; Lachenaud *et al.*, 2002). Population breeding schemes are currently under way in the Ivory Coast using an Upper Amazon population and another composed of Lower Amazon and Trinitarios genotypes, and results of the first cycle of selection have been reported (Lachenaud *et al.*, 2002). In Brazil, recurrent selection has been adopted using a Comstock II design with two popula-

tions (Upper Amazonian wild genotypes and domesticated Forastero and Trinitario genotypes) in order to estimate the general combining ability of each parent and genetic similarities among genotypes using molecular markers (Pires *et al.*, 2000a). Intrapopulation crosses would be the basis for the next cycle of selection, while interpopulation crosses would allow selection of clones and hybrid varieties in each generation.

2. Molecular Genetics

2.1. Gene cloning

Cacao has a small haploid genome, estimated to be approx. 415 Mbp (Figueira *et al.*, 1992) or 388 Mbp (Lanaud *et al.*, 1992) and approx. three times the *Arabidopsis* genome size. The genome size, measured by reassociation kinetics, has been estimated to be 201 Mbp (Couch *et al.*, 1993), with 36% of G + C, a low degree of methylation and a small amount of highly repetitive DNA. The small genome size suggests that map-based cloning should be effective. The cacao chloroplast genome has been estimated to be approx. 96 kbp (Chung, 1988) to 109 kbp (Yeoh *et al.*, 1990).

Only a few cacao genes have been cloned, based on cDNA and/or genomic library screening: the 21 kDa trypsin inhibitor gene (Spencer and Hodge, 1991; Tai *et al.*, 1991), the 67 kDa vicilin-like storage protein gene (McHenry and Fritz, 1992; Spencer and Hodge, 1992) and the chitinase I gene (Snyder-Leiby and Furtek, 1995). A 2S albumin storage peptide has been identified and characterized, and the corresponding full-length cDNA has been cloned (Kochhar *et al.*, 2001). Similarly, cDNAs of two seed aspartic proteinases (TcAP1 and TcAP2), believed to be associated with the development of flavour precursors, have also been cloned and characterized (Laloi *et al.*, 2002). Sequences for other seed proteins (15.8 kDa and 16.9 kDa oleosins, and carboxypeptidase type III) have been deposited at the GenBank of the National Center for Biotechnology Information

(NCBI) (<http://www.ncbi.nlm.nih.gov>) by the Centre de Recherche Nestlé.

Sequencing has been mostly based on amplification by universal primers for conserved nuclear and plastid genes for phylogenetic studies, or by primers developed for conserved motifs, e.g. those for resistance-gene homologues (RGHs) (Kuhn *et al.*, 2001, 2003). The nuclear genes, arginine decarboxylase (*spe2*) (Galloway *et al.*, 1998), the 18S rDNA (Soltis *et al.*, 1999) and the trypsin inhibitor gene (Silva *et al.*, 2002), together with plastid genes, H⁺-transporting ATP synthase (*atpB*), ribulose 1,5-bisphosphate carboxylase large subunit (*rbcl*) (Alverson *et al.*, 1998, 1999; Bayer *et al.*, 1999) and the nicotinamide adenine dinucleotide dehydrogenase (NADH) subunit F (*ndhF*) (Whitlock *et al.*, 2001), have been amplified and sequenced from cacao and other *Theobroma* species, and used in phylogenetic studies. Forty-nine microsatellite loci have been characterized from genomic sequences (Lanaud *et al.*, 1999b), and more are currently under development at the Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD).

Lanaud (2002) using a candidate-resistance gene approach (resistance and defence gene homologues), cloned and sequenced 42 RGHs and mapped 22 of them on the cacao consensus linkage map, and a few co-localized with *Phytophthora* resistance. A similar approach has been adopted by Kuhn *et al.* (2001, 2003) to amplify RGHs using the degenerate primers RG1 and RG2 proposed by Aarts *et al.* (1998) based on *Arabidopsis* sequences, and the primer combination PLOOPGA + GLPL6 developed for lettuce (Shen *et al.*, 1998), using genomic DNA from seven cacao genotypes. To date (October 2002), 84 RGH sequences have been deposited at the GenBank of NCBI, including six sequences classified as RGH5 and four RGH1, plus 65 functional nucleotide binding site-leucine rich repeats and nine non-functional, classified into at least 11 TcRGH categories (Kuhn *et al.*, 2001).

Attempts are under way to clone the cacao homologues for the *Brassica* S-locus receptor kinase gene, responsible for the pollen-stigma recognition reaction in sporo-

phytic self-incompatible systems, based on primers designed to anchor at the highly conserved transmembrane domain (Ford and Wilkinson, 2002).

2.2. Marker-assisted selection

Genetic studies of cacao have been hampered by the long juvenile cycle, by the high costs associated with the maintenance and evaluation of large populations for extended periods and by heterozygosity. The development of genomic maps based on molecular markers would facilitate early selection, using the detection of genomic regions associated with desirable traits as indirect selection criteria. Genomic maps are developed based on joint segregation of molecular markers and characters of interest, with oligo- or polygenic inheritance, in families derived from controlled crosses (Staub *et al.*, 1996). To maximize information obtained from a mapping population, parental genotypes should be important for the breeding programme and present enough molecular polymorphism to enable mapping and contrast for various quantitative and qualitative characters, e.g. yield components, disease and pest resistance, fat content and quality and possibly flavour. Annual crops are generally mapped using F_2 or F_3 populations, back-crosses or recombinant inbred lines, derived from crosses between inbred lines or from duplicated haploids (Kochert, 1994).

In cacao, widely available F_1 hybrid varietal trials, derived from crosses between two heterozygous genotypes, have been more frequently used to develop linkage maps (Lanaud *et al.*, 1995; Risterucci *et al.*, 2000a; Flament *et al.*, 2001; Clément *et al.*, 2002a,b), exploiting the 'pseudo-testcross' configuration for various loci (Grattapaglia and Sederoff, 1994). A map is generated for each heterozygous parent, based on segregation of co-dominant markers (such as RFLPs, simple sequence repeats (SSRs) and isozymes); both maps can be joined or compared to an established reference map (Risterucci *et al.*, 2000a). The more abundant dominant markers (RAPDs and amplified fragment length polymorphisms (AFLPs))

are used to saturate this map. Often, a highly heterozygous genotype has been crossed with a more homozygous parent, e.g. the albino mutant 'Catongo'. Alternatively, a back-cross population, derived from a cross between an F_1 plant and a more homozygous recurrent parent, has been used (Crouzillat *et al.*, 1996), while, in other specific cases, F_2 populations have been specifically developed and used to identify quantitative trait loci (QTLs) associated with flavour and seed quality (Crouzillat *et al.*, 2001) and witches' broom resistance (Queiroz *et al.*, 1998).

The first cacao linkage map has been developed for 'UPA402' $\times$ 'UF676' progeny (Lanaud *et al.*, 1995). This map has ten linkage groups, containing 193 marker loci, including five isozymes; 160 RFLPs, derived from 101 cDNA and 55 genomic probes, and four known genes; and 28 RAPD loci, covering 759 cM. The average distance between markers is 3.9 cM. Based on the estimated haploid genome size, the physical size ranges from 511 to 547 kbp/cM, possibly favouring gene cloning by chromosome walking. The map was originally developed based on 100 individuals in the Ivory Coast. This linkage map was later saturated with additional markers (Risterucci *et al.*, 2000a), mainly 191 AFLP and 20 microsatellite loci, plus another 18 RFLP loci (ten genomic probes, three cDNAs, two known genes, three telomeric probes) and 30 RAPD loci, comprising 424 markers, covering 885.4 cM, with an average spacing between two markers of 2.1 cM. The mapping population has been increased to 181 individuals in the Ivory Coast, and this map is now considered the consensus linkage map and reference for linkage group or chromosome numbering for cacao (Clément *et al.*, 2001).

Crouzillat *et al.* (1996) developed a second linkage map for cacao, using a back-cross population of 131 plants, derived from a cross between a single F_1 tree from 'Catongo' $\times$ 'Pound 12' to a recurrent 'Catongo'. The final back-cross map was comprised of 140 markers, including 79 RFLP loci, 59 RAPDs and two morphologic loci (self-compatibility and anthocyanin synthesis) and covered 944 cM in ten linkage groups (Crouzillat *et al.*, 2000a). A genomic map has also been

developed for the related F_1 population from the cross 'Catongo' $\times$ 'Pound 12'. The F_1 map displayed 162 markers (125 RFLPs, 23 RAPDs, 13 AFLPs and one morphological marker) and covers 772 cM in ten linkage groups (Crouzillat *et al.*, 2000a).

Various linkage maps have been developed, and used mostly to identify genomic regions associated with yield and its components, plant vigour and resistance to various *Phytophthora* species and strains (Table 21.1.1).

The total amount of dry fermented cacao seed produced by a tree is a function of the total number of pods harvested and the average pod weight, determined by the number of seeds per pod and single seed weight. Yield per hectare depends on planting density, which is related to tree vigour and agronomic practices, including soil fertility. Average number of pods is significantly correlated ($r = 0.96$) with a 15-year average yield of the F_1 'Catongo' $\times$ 'Pound 12' progeny (Crouzillat *et al.*, 2000a), or based on the 9-year cumulative yield ($r = 0.89$ – 0.95) for three progenies derived from crosses between the Trinitario genotypes 'DR1' or 'S52', or the Upper Amazon Forastero 'IMC78' with 'Catongo' (Clément *et al.*, 2002a). No correlation has been observed between seed yield and other yield components (Crouzillat *et al.*, 2000a; Clément, 2001; Clément *et al.*, 2002a). Yield is correlated with mature tree vigour, as estimated by trunk girth (Crouzillat *et al.*, 2000a; Clément *et al.*, 2002a) and canopy size (Clément *et al.*, 2002a).

Based on yield data collected for 15 years, ten QTLs have been identified in eight linkage groups for the F_1 'Catongo' $\times$ 'Pound 12' progeny in Costa Rica (Crouzillat *et al.*, 2000a). Two genomic regions, on chromosomes 4 and 5, each explaining *c.* 20% of the total phenotypic variance, were detected as early as 4 years after planting, and have been consistently detected for another 12 years. In Ivory Coast, three QTLs for seed yield have been identified for each parental genotype ('DR1', 'S52' and 'IMC78') in six distinct linkage groups (Clément, 2001; Clément *et al.*, 2002a). These QTLs have differed for expression over the years, with the QTLs from

'IMC78' detected at a later stage (10 years after planting), but for a longer period, than those from the Trinitarios. A specific QTL, located on chromosome 5 of 'IMC78' ($r^2 = 11.2$) was detected repeatedly between the 10th and 16th year after planting. This stable yield QTL detected for 'IMC78' on chromosome 5 was co-localized with QTLs detected in 'Pound 12' in Costa Rica (Crouzillat *et al.*, 2000a), probably because of the common genetic origin of the two genotypes (Clément *et al.*, 2001). Common QTLs for yield were also detected on chromosome 1 for the Trinitario genotypes 'S52' (Clément *et al.*, 2002a) and 'UF676' (Lanaud *et al.*, 2000). Identical QTLs were identified for average seed yield and for pod number in all progenies (Crouzillat *et al.*, 2000a; Clément *et al.*, 2002a).

A major QTL for pod weight in 'IMC78', responsible for 43.5% of the phenotypic variation, was detected on chromosome 4, at close proximity to a similar QTL for the Trinitario genotypes 'DR1' and 'T60/887' (Clément *et al.*, 2001). A QTL for pod index, defined as the number of pods required to produce a kilogram of cacao seeds (function of pod weight), has also been identified on chromosome 4 of 'Pound 12' (Crouzillat *et al.*, 2000a). Similarly, QTLs for pod weight have been co-localized on chromosome 1 for the Trinitario genotypes 'DR1' and 'UF676' (Clément *et al.*, 2002a).

Three QTLs for seed weight are on chromosomes 4, 5 and 9 of 'Pound 12' (Crouzillat *et al.*, 2000a). The most significant QTLs for seed weight in 'S52' and 'IMC78', explaining 16.2% and 13.6% of phenotypic variation, respectively, have been detected on the same region of chromosome 4 as in 'Pound 12' (Clément *et al.*, 2001). Another co-localization of QTLs includes the region of chromosome 9 containing a QTL for seed weight identified in 'S52' (Clément *et al.*, 2001) and in 'UF676' (Clément *et al.*, 2002b).

Seed weight is negatively correlated with the number of seeds per pod in 'Pound 12' (Crouzillat *et al.*, 2000a). A major QTL for number of seeds per pod is located on chromosome 4, together with another QTL on chromosome 6 (Crouzillat *et al.*, 2000a). The number of seeds per pod is related to the

Table 21.1.1.1. Genomic maps established for identification of genomic regions associated with yield components, plant vigour and *Phytophthora* resistance.

Progenies	No. of plants	No. of markers	No. of linkage groups	Map length (cM)	Reference
UPA 402 × UF 676	181	473	10	887	Risterucci <i>et al.</i> , 2000b
T60/887 × IFC 2	(59)				
T60/887 × IFC 5	(56) 112	198	11	793	Flament <i>et al.</i> , 2001
Catongo × Pound 12	55	162	12	772	Crouzillat <i>et al.</i> , 2000b
Catongo × (Catongo × Pound 12)	131	140	10	944	Crouzillat <i>et al.</i> , 2000a
ICS 84 × UPA 134	62	224	15	548	Flament, 1998
SNK 10 × UPA 134	78	151	16	617	Flament, 1998
SNK 413 × IMC 67	58	119	15	419	Flament, 1998
DR 1 × Catongo	107	192	9	653	Clément <i>et al.</i> , 2002a,b
S 52 × Catongo	101	138	11	589	Clément <i>et al.</i> , 2002a,b
IMC 78 × Catongo	128	223	10	721	Clément <i>et al.</i> , 2002a,b
(Scavina 6 × H) × IFC 1	151	202	10	724	Risterucci <i>et al.</i> , 2000b
IMC 57 × Catongo	155	235	12	1427	Motilal <i>et al.</i> , 2002

number of ovules, and major genetic differences have been observed between the distinct cacao morphogeographic groups (Clément *et al.*, 2002b); however, the number of seeds per pod is not correlated with the number of ovules (Crouzillat *et al.*, 2000a). A QTL for ovule number has been identified on chromosome 2 for the 'Catongo' × 'Pound 12' progeny (Crouzillat *et al.*, 2000a). Similarly, QTLs for ovule number have been identified at the same linkage group in 'S52' (Clément, 2001; Clément *et al.*, 2002b). Another QTL for ovule number has been identified at the same region of chromosome 4 in 'IMC78' and 'DR1'. Various QTLs for seed size, seed weight and number of ovules are co-localized, especially on chromosome 4 (Crouzillat *et al.*, 2000a; Clément *et al.*, 2001, 2002b). Crouzillat *et al.* (2000a) identified the locus for self-compatibility (*Autoc*) at chromosome 4, a trait associated with domestication of cacao (Cope, 1976). The number of pods and yield are directly affected by self-compatibility. Among the various QTLs identified for seed size measurements, one particular region of chromosome 4 contained important QTLs for seed length, width, length to thickness ratio, seed weight and ovule number detected for three parental genotypes belonging to distinct morphogeographic groups (Trinitarios 'DR1' and 'S52', and Forastero 'IMC78') (Clément *et al.*, 2002a,b). The favourable alleles for larger size in both Trinitario genotypes had a Forastero origin.

Selection of hybrid varieties has been based on field evaluation for 4 to 8 years (Lockwood and Pang, 1993), and there has been a trend to select for juvenile vigour, which is assumed to be associated with high yield at maturity. Attempts to correlate yield with stem diameter have failed (Hadley and Yapp, 1993). Average yield based on 15-year data has been significantly correlated ($r = 0.56$) with trunk diameter in the 'Catongo' × 'Pound 12' F_1 population (Crouzillat *et al.*, 2000a). Similarly, average yield of three F_1 populations ('S52', 'DR1' and 'IMC78' crossed with 'Catongo') is significantly correlated with mature tree vigour, estimated by trunk circumference and canopy width, but not with stem diameter, estimated in the 2nd

year after planting, except for the 'S52' progeny (Clément *et al.*, 2002a). Stem diameter and trunk circumference are significantly correlated.

The QTLs for stem diameter, identified on chromosome 2 and 7 for the 'Catongo' × 'Pound 12' × 'Catongo' BC_1 population, 2 years after planting (Crouzillat *et al.*, 1996) differed from the QTLs identified for trunk diameter at chromosomes 1, 4 and 5 for the F_1 population, estimated 6 years after planting (Crouzillat *et al.*, 2000a). Similarly, the QTLs for height of first jorquette differed for both populations. A common QTL for stem diameter and height of jorqueting has been identified on linkage group 7 for the BC_1 population (Crouzillat *et al.*, 1996) and on linkage group 4 for the F_1 population (Crouzillat *et al.*, 2000a). The significant QTLs identified for canopy width for 'DR1' and 'IMC78' are co-localized with yield QTLs, while QTLs for stem diameter and trunk circumference are closely located to yield QTLs in 'IMC78' (Clément *et al.*, 2002a).

Black pod or *Phytophthora* pod rot is the most important disease of cacao, causing losses as high as 30% under favourable climatic conditions (Lanaud *et al.*, 2000; Flament *et al.*, 2001). Control measures are costly, and include removal of infected pod and chemical spray with cupric fungicides. Genetic resistance is an attractive long-term control method, with low cost to growers and less impact on the environment. Resistance to *Phytophthora* seems to be polygenic with additive inheritance (Tan and Tan, 1990; Warren and Pettitt, 1994; Cilas *et al.*, 2000). Screening for resistance has been conducted based on natural pod rot losses (Luz *et al.*, 1997; Cilas *et al.*, 2000), by artificial inoculation of attached pods and by inoculation of leaf discs (Nyassé *et al.*, 1995).

To identify genomic regions associated with *P. palmivora*, *P. megakarya* and *P. capsicii* resistance, 12 genomic maps have been published for populations in Ivory Coast, Costa Rica, Cameroon, Trinidad and France (Table 21.1.1). Resistance has been evaluated accordingly: (i) by percentage of pod rot rate under field conditions for *P. megakarya* (Flament, 1998) and *P. palmivora* (Lanaud *et al.*, 2000; Clément, 2001; Clément *et al.*, 2001,

2002a; Flament *et al.*, 2001); (ii) by artificial inoculation of leaf discs using one strain of *P. megakarya* (Flament, 1998) or one strain of *P. palmivora* (Lanaud *et al.*, 2000; Flament *et al.*, 2001; Motilal *et al.*, 2002); (iii) by artificial inoculation of leaf discs using two strains of *P. megakarya*, *P. palmivora* and *P. capsicii* (Risterucci *et al.*, 2000b); (iv) by pod inoculation with wounding (Flament, 1998; Flament *et al.*, 2001); and (v) by pod inoculation without wounding (Crouzillat *et al.*, 2000b).

No correlation between pod rot rate and resistance by leaf disc inoculation with *P. palmivora* was observed for the 'UPA402' × 'UF676' progeny (Lanaud *et al.*, 2000). Detection of QTLs for resistance estimated by leaf disc inoculation was limited by the lack of precision and stability of this method, while a significant QTL associated with field rot rate was detected on chromosome 1 for both parents. Resistance estimated by leaf disc and pod inoculations with *P. palmivora* is weakly correlated with field pod rot rate, measured over 2 years for the 'T60/887' × 'IFC5/IFC2' progenies (Flament *et al.*, 2001). Correlation between inoculation of pods and leaf discs is not strong. The leaf inoculation is highly influenced by the environment, with low reproducibility and a poor correlation with yield losses. A significant QTL for resistance to *P. palmivora* has been identified for pod rot rate on chromosome 10, two QTLs based on the leaf test (chromosomes 6 and 3) and two by pod inoculation (chromosomes 2 and 6). No QTL for resistance is common for either method, suggesting either that each evaluation method accounted for a distinct genetic mechanism of resistance or that the size of the progeny and/or the low reproducibility of the tests is not accurate enough to detect all QTLs involved (Flament *et al.*, 2001). Similar results have been obtained for tests with *P. megakarya* (Flament, 1998). QTLs have been identified for resistance to *P. megakarya* on chromosome 9 evaluated by leaf inoculation and on chromosome 2 by pod inoculation. Lack of reproducibility in trials of leaf disc inoculation with *P. palmivora* has also been observed by Motilal *et al.* (2002).

Six QTLs for *Phytophthora* resistance, evaluated by artificial pod inoculation, have been

identified on five linkage groups for the 'Catongo' × 'Pound 12' F₁ and the BC₁ progenies (Crouzillat *et al.*, 2000b). Only one QTL (on chromosome 9) was common to both populations with a major effect on the F₁, since, for the other three QTLs, both parents are homozygous and did not segregate in the F₁ population, while, for the other two QTLs, the F₁ plant chosen for back-crossing did not have the favourable allele. There has been no major co-localization of these QTLs with the results of Flament (1998) and Flament *et al.* (2001), except for a minor QTL identified at chromosome 2, similarly to one found based on pod inoculation.

Based on inoculation of leaf discs of the ('Scavina 6' × unknown) × 'IFC 1' progeny, using two strains of each of three *Phytophthora* species (*P. palmivora*, *P. megakarya*, *P. capsici*), eight QTLs for resistance have been detected, four on chromosome 5 and one each on chromosomes 1, 3, 4 and 6 (Risterucci *et al.*, 2000b). Three QTLs are significant for more than one species, but none is significant for all of them, while only three QTLs are significant for resistance to both strains of *P. palmivora*. There is no correspondence between these QTLs and those identified by Flament *et al.* (2001) for *P. palmivora* and *P. megakarya* (Flament, 1998) using similar inoculation conditions. Motilal *et al.* (2002) identified three major QTLs (chromosomes 1, 9 and 3 or 8) and four minor QTLs (chromosomes 1, 2, 4 and 9) that co-localized with QTLs detected in other studies, based on leaf inoculation with *P. palmivora* of 'IMC 57' × 'Catongo' in Trinidad.

For percentage of rotten pods under natural conditions in Ivory Coast, one QTL has been identified at chromosome 10 with a log of odds (LOD) score of 4.2 in the 'T60/887' × 'IFC2/IFC5' progenies for a 2-year harvest period (5th and 6th year after planting) (Flament *et al.*, 2001), while Clément *et al.* (2002a) detected significant QTLs for 'DR1' and 'IMC78', both in the same region of chromosome 4, based on harvest data between the 8th and 13th year after planting. These QTLs are co-localized with vigour traits, e.g. trunk circumference and canopy width.

Most of the QTLs for yield, plant vigour and resistance to *Phytophthora* have been identified from pre-existing hybrid trials with a limited number of individuals, and often involved parental genotypes without the ideal contrast for traits or enough DNA polymorphism. The occurrence of illegitimate individuals is a common problem in these trials, reducing the number of scorable plants (Flament, 1998; Crouzillat *et al.*, 2000a; Clément, 2001; Clément *et al.*, 2002a,b). The accuracy of QTL identification is dependent on the number of individuals available in the mapping populations, and therefore it is advisable to develop specific populations, with a minimum of 200 individuals, as suggested by Clément *et al.* (2000). The use of interrelated populations, derived from factorial or diallel mating designs, with common parental genotype, can facilitate QTL identification (Clément *et al.*, 2001). Other problems affecting the accuracy of QTL identification include lack of sufficient replications and the methods used for evaluating resistance or subjective traits, e.g. flavour (Crouzillat *et al.*, 2001; Bucheli *et al.*, 2002). Nevertheless, some of the QTLs associated with yield, vigour and resistance to *Phytophthora* have been identified at the same chromosome region for various unrelated genotypes, under different environmental conditions, with some stability over several years and/or locations, and could be good candidates for marker-assisted selection (MAS).

Because of the cost and/or low precision of the identified QTLs, their direct use for MAS is unlikely at this point; however, Lanaud (2001) proposed that QTLs for resistance to *Phytophthora* could be used to develop genotypes homozygous for resistance alleles by selfing and screening with molecular markers and to accumulate various resistance genes by crossing genotypes with different resistance QTLs. Clément *et al.* (2001) proposed two main approaches for joint application of MAS: (i) monitoring the accumulation of favourable alleles during back-cross breeding; and (ii) use of marker-assisted recurrent selection (MARS) to increase the frequencies of favourable alleles of populations under recurrent selection. The

use of markers should be combined with phenotypic evaluation in a selection index (Lanaud, 2001). Crouzillat *et al.* (2000a) simulated the use of markers in yield selection in comparison to phenotypic or the combination of phenotypic evaluation with molecular markers based on two QTLs for yield. The use of molecular markers alone or in combination with phenotypic selection was more effective.

3. Micropropagation

Cacao has been recalcitrant with respect to *in vitro* propagation, and there is no continuous shoot growth after bud break (Figueira and Janick, 1995). Passey and Jones (1983) initiated research in this area; however, only a single study has appeared recently (Lardet *et al.*, 1998). Micropropagation of related *Sterculia urens* from cotyledonary nodes (Purohit and Dave, 1996) and from nodal explants of elite selections (Sunnichan *et al.*, 1998) has been reported. Dossa *et al.* (1994) induced bud break and elongation of *Cola nitida* microcuttings that originated from nursery-grown plants, but shoots failed to root. *In vitro* culture of cupuassu has been attempted to propagate superior genotypes, especially a seedless mutant identified in Brazil with high pulp yield (Velho *et al.*, 1990).

Three types of explant have been used for shoot tip and nodal culture: axillary buds from orthotropic and plagiotropic shoots and from cotyledonary nodes. Most reports have described only the initiation of cultures to bud break (Dufour and Dublin, 1985; Bertrand, 1987), with few reports describing bud elongation (Passey and Jones, 1983; Janick and Whipkey, 1985; Litz, 1986; Bertrand, 1987). Significant growth of axillary shoots has rarely been observed (Passey and Jones, 1983; Flynn *et al.*, 1990; Figueira *et al.*, 1991, 1994). The effect of genotype on bud break and flushing has not been reported.

Optimal conditions for growth include high temperature and relative humidity (RH). Axillary buds from orthotropic or plagiotropic shoots from greenhouse-grown or field-grown plants are generally difficult to disinfect, and fungicide must be applied in

the field before collecting (Legrand and Mississo, 1986). Disinfestation involving several steps is generally necessary (Bertrand, 1987; Lardet *et al.*, 1998). Water potential of explants decreases significantly after disinfestation, but no change in water content occurs; electrical conductivity is significantly higher in the water of disinfested explants (Aguilar, 1996).

After bud break, elongation has rarely been achieved. The physiological state of shoots from which explants have been collected influences establishment and development (Bertrand, 1987; Lardet *et al.*, 1998). Lardet *et al.* (1998) demonstrated that buds from dormant shoots respond better than those collected during active meristem growth, while Passey and Jones (1983) recommended axillary buds from actively flushing shoots. The position of nodes relative to the shoot apex can also affect establishment; axillary buds distal from the shoot tip are more likely to respond (Flynn *et al.*, 1990; Lardet *et al.*, 1998).

There is no consensus about the effects of plant growth regulators on bud break and elongation. For axillary buds, bud break has been initiated with cytokinin alone (Passey and Jones, 1983), with auxin and cytokinin (Dufour and Dublin, 1985; Legrand and Mississo, 1986; Bertrand, 1987; Lardet *et al.*, 1998) and with no plant growth regulators (Flynn *et al.*, 1990). For cotyledonary nodes, bud break has been achieved with auxin alone (Legrand *et al.*, 1984), with cytokinin alone (Janick and Whipkey, 1985) and with auxin and cytokinin (Legrand and Mississo, 1986).

Murashige and Skoog (1962) (MS) basal medium has been preferred, although Flynn *et al.* (1990) and Figueira *et al.* (1991, 1994) used woody plant medium (WPM) (Lloyd and McCown, 1980). Dufour and Dublin (1985) utilized reduced-strength MS. Bud break and/or shoot elongation are stimulated in liquid medium, by increased culture vessel volume, activated charcoal (Dufour and Dublin, 1985), frequent medium transfer (Legrand and Mississo, 1986), glucose (Legrand *et al.*, 1984) and fructose (Figueira *et al.*, 1991). Elevated CO₂ (20,000 ppm) and photosynthetic photon flux density (150–200 $\mu\text{mol}/\text{m}^2/\text{s}$) stimulate bud elongation and leaf production from axillary shoots, single-

node cuttings and microcuttings from mature plants (Figueira *et al.*, 1994). These conditions are partly due to increased net photosynthesis (Figueira *et al.*, 1994).

Bud proliferation occurred from plagiotropic buds (Passey and Jones, 1983). Litz (1986) obtained explants with six buds after 3 months, using medium supplemented with benzyladenine (BA) and zeatin, but limited bud elongation occurred. Janick and Whipkey (1985) obtained bud proliferation from cotyledonary nodes, and axillary shoots could grow while attached to the original node, but failed to grow when detached from the explant. Figueira *et al.* (1991) initiated up to six buds per node using thidiazuron (TDZ) and, under high CO₂ levels and photon flux densities, the axillary shoots elongated and grew when detached from the explant.

Rooting has been reported for single-node cuttings (Passey and Jones, 1983; Dufour and Dublin, 1985; Flynn *et al.*, 1990) and cotyledonary nodes (Legrand *et al.*, 1984). Indolebutyric acid (IBA) has been used alone or with naphthaleneacetic acid (NAA) (Passey and Jones, 1983), or with sucrose and activated charcoal (Dufour and Dublin, 1985; Flynn *et al.*, 1990). Aguilar (1996) compared the rooting of axillary shoots of two genotypes under various conditions, including IBA concentration, period of exposure to IBA, sucrose concentration and liquid medium. Responses to these treatments and to acclimatization were genotype-dependent.

4. Somatic Cell Genetics

4.1. Regeneration

4.1.1. Somatic embryogenesis

Embryogenic cultures were first reported from immature zygotic embryos by Esan (1974). This was later confirmed by Pence *et al.* (1979, 1980, 1981a,b), Kong and Rao (1982), Tsai *et al.* (1982), Kononowicz and Janick (1984a,b), Kononowicz *et al.* (1984), Tahardi (1984), Wang and Janick (1984, 1985), Wen *et al.* (1984), Wright *et al.* (1984), Esan (1985a,b), Elhag *et al.* (1987, 1988), Lanaud (1987c), Adu-Ampomah *et al.* (1988), Duhem *et al.* (1989),

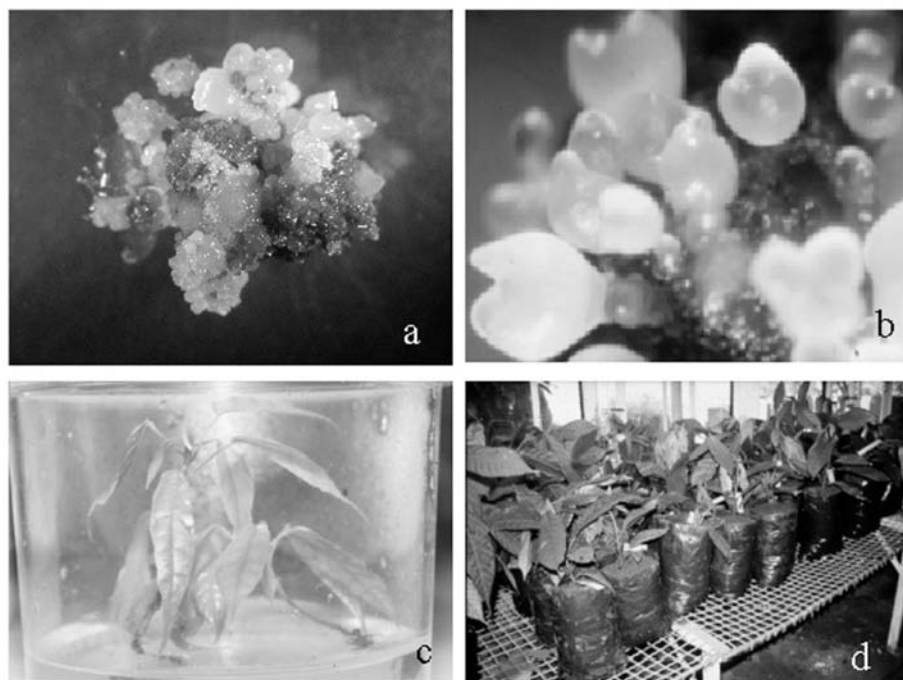


Fig. 21.1.1. Primary and secondary embryogenesis according to Li *et al.* (1998). (a) Embryogenic cultures derived from staminodes on ED medium; (b) somatic embryo development after 4 weeks of culture on ED medium; (c) *in vitro* plant derived from somatic embryo; (d) somatic plantlets.

Santos and Machado (1989) and Aguilar *et al.* (1992). Somatic embryogenesis from immature zygotic embryos of cupuassu has been reported (Janick and Whipkey, 1988).

Induction. Induction of embryogenic cultures from sporophytic tissues has been investigated, using various explants and media (Litz, 1986; Söndahl *et al.*, 1988, 1989, 1993; Chatelet *et al.*, 1992; Figueira and Janick, 1993). Litz (1986) induced embryogenic cultures from leaf explants; however, the frequency was very poor, and the somatic embryos aborted rapidly. Induction of embryogenic cultures from the nucellus was described by Söndahl *et al.* (1988, 1989, 1993), Chatelet *et al.* (1992) and Figueira and Janick (1993); however, the response occurred at low frequency and was genotype-dependent. Flower parts have also been used as explants (Söndahl *et al.*, 1988; Lopez-Baez *et al.*, 1993; Alemanno *et al.*, 1996, 1997). Staminodes are the most responsive explant (Alemanno *et al.*, 1996, 1997; Li *et al.*, 1998);

however, Lambert *et al.* (2002) claimed that petal bases from flowers of several genotypes are more embryogenic than staminodes. Embryogenic cultures are induced from explanted flower buds after 3 weeks on semi-solid MS medium containing amino acids, coconut water, 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin and sucrose in darkness (Lopez-Baez *et al.*, 1993). Li *et al.* (1998) utilized a two-step induction of 2 weeks each, on two specific media (Fig. 21.1.1): primary callus growth (PCG) and secondary callus growth (SCG). The major modifications were the use of Driver and Kuniyuki (1984) (DKW) salts and vitamins in PCG and WPM and B5 (Gamborg *et al.*, 1968) vitamins in SCG, no coconut water in PCG, TDZ in PCG and glucose as the carbon source. This protocol has been efficient for induction of embryogenic cultures for many genotypes, although there is still a large genotypic effect (Lopez-Baez *et al.*, 1993; Alemanno *et al.*, 1996; Li *et al.*, 1998; Tan *et al.*, 2002; Table 21.1.2). The percentage of

Table 21.1.2. Somatic embryogenesis from staminodes of different cocoa genotypes.

Genotype	Reference	Method used	% Embryogenic explants	No. of somatic embryos per embryogenic explant	Normal somatic embryo rate (%)	% Conversion
EET 48	Lopez-Baez <i>et al.</i> (1993)	Lopez-Baez <i>et al.</i> (1993) Petals and staminodes	From 1.3 to 18.7	Not determined	Not determined	66.7
EET 64						
EET 94						
EET 228						
CC 260						
NA 79	Alemanno <i>et al.</i> (1996)	Lopez-Baez <i>et al.</i> (1993) Staminodes	81.5	Not determined	Not determined	Not determined
UPA 603			43.7			
T 85/799			34.4			
R 43			0.0			
R 106			0.0			
Catongo	Li <i>et al.</i> (1998)	Li <i>et al.</i> (1998) Staminodes	5.8	4.8	Not determined	Not determined
Laranga			85.6	13.5		
Matina			22.3	4.7		
Sca 6–1			100	45.7		
Sca 6–2			98.3	40.9		
Sca 6 × ICS1 No. 1			23.2	8.1		
Sca 6 × ICS1 No. 3			31.7	5.8		
TSH 1112			0.8	27		
RB 48			5.0	6.4		
EET 400			0.8	1.0		
Unknown Criollo			6.7	4.3		
Pentagona F1			20.6	2.0		
ICS 1			14.2	3.9		
ICS 16			45.8	6.4		
ICS 39			10.0	2.0		
ICS 67			0.8	42.0		
Pound 7			7.2	2.8		
Tomate Ceplac			1.7	2.0		
UF 613			45.6	5.6		

Continued

Table 21.1.2. Continued.

Genotype	Reference	Method used	% Embryogenic explants	No. of somatic embryos per embryogenic explant	Normal somatic embryo rate (%)	% Conversion
Scavina-6	Li <i>et al.</i> (1998)	Alemanno (unpublished data) Staminodes	72.1 ± 10.1	43 ± 42.7	37.9	11.3 ± 20.4
NA 79			41.7 ± 10	16.3 ± 3.3	43.6	20.7 ± 7.3
GU 143 A			27.1 ± 12.6	21.3 ± 16.2	93	12.2 ± 16.2
T 85/799			22.5 ± 21.4	11.3 ± 3.2	67.3	24.5 ± 7.6
IMC 67			18.6 ± 4.5	33.1 ± 31.8	35.3	37.5 ± 44.5
KER 1			15.1 ± 9.6	17.2 ± 8.9	58.7	1 ± 2.8
GF 23			10.0	9 ± 4.3	22.2	18.3 ± 18.5
NA 32			9.2 ± 5.7	35.2 ± 24.1	56.3	12.2 ± 16.2
Sca-12			8.0	4.0	25	0.0
IFC 705			7.3 ± 0.9	8.7 ± 5.3	46	18.4 ± 22.2
IFC 5			6.1 ± 7.4	4.1 ± 0.1	56.1	41.7 ± 16.7
CC 222			3.9 ± 3.	3.5 ± 1.4	48.6	6.7 ± 9.4
R 48			2.8 ± 3.6	6.2 ± 3.8	82.3	9.7 ± 7.1
UF 667			0.6 ± 1.7	6	83.3	16.7
DR 38						
SPEC 138.8						
PA 120						
PA 121			0.0	0.0	0	0
ICS 39						
ICS 48						
ICS 95						
LAF 1						
COCA3370-5	Lopez-Baez <i>et al.</i> (1993) Modified	Tan <i>et al.</i> (2002) Staminodes	18.2	Not determined	Not determined	Not determined
CAB 64			4.8			
AMAZ 15/15			4.7			
AMAZ 12			2.6			
IMC 96			2.2			
Scavina-6			1.7			
GU 147H			1.6			
SIAL 93			0.9			
Scavina-24			0.0			

embryogenic explants of various genotypes ranges from 0 to 100%. The number of somatic embryos per responsive staminode varies from one to 45. Lambert *et al.* (2002) reported up to 60 somatic embryos per embryogenic petal base. Lopez-Baez *et al.* (2001) and Tan *et al.* (2002) also reported improved embryogenic response of two genotypes with DKW salts.

According to Alemanno *et al.* (1996) and Maximova *et al.* (2002), embryogenic cultures originate from perivascular and epidermal cells of staminodes. Embryogenic cultures are initially nodular, and consist of meristematic zones with actively dividing cells, which acquire distinctive proembryonic morphology, often in isolation from the surrounding cells. The proembryonic cell clusters are somatic proembryos, which acquire a protoderm and procambial bundles (globular stage). Subsequently, heart-shaped and cotyledonary stages of development are recognizable. For 100 cultured staminodes, approx. 129 plants can be produced for 'Scavina 6', and only two plants for 'R48' (Table 21.1.2).

Maintenance. Figueira and Janick (1995) demonstrated that friable embryogenic cultures can be maintained for long periods. Occasional secondary somatic embryogenesis has been observed from cacao immature zygotic embryos (Pence *et al.*, 1979; Novak *et al.*, 1985; Duhem *et al.*, 1989) and from somatic embryos (Alemanno *et al.*, 1996; Li *et al.*, 1998).

Secondary somatic embryogenesis from primary somatic embryos can be sustained by slicing cotyledons of primary somatic embryos into pieces of approx. 4 mm² (Maximova *et al.*, 2002) and culturing them on SCG medium for 14 days, followed by transfer to plant growth regulator-free medium (Li *et al.*, 1998; Fig. 21.1.1). Secondary somatic embryogenesis can be achieved for most genotypes. According to Maximova *et al.* (2002), secondary embryos develop from single cells of the protoderm. Guiltinan and Maximova (2001) concluded that one staminode of 'Scavina 6' can produce an average of 17 normal somatic embryos, while one primary somatic embryo cotyledon explant could produce an average

of 70 normal embryos. The efficiency of secondary somatic embryogenesis is affected by the age of the primary somatic embryos, with the maximum number of secondary somatic embryos produced 24 weeks after primary culture induction. Fontanel *et al.* (2002a) maintained embryogenic cultures from 18 genotypes for six subcultures.

A temporary immersion system (*réceptif à immersion temporaire aéropulsée* (RITA)) (Alvard *et al.*, 1992) has been tested for secondary somatic embryogenesis using the procedure of Maximova *et al.* (2002) (Alemanno *et al.*, 2002; Fig. 21.1.2). The RITA system increased the number of cacao somatic embryos produced per explant and improved somatic embryo development. Somatic embryo development, measured 6 weeks after transfer, was significantly increased with no subculture, especially with the 4 × 1 min/24 h cycle.

Maturation. Somatic embryo development occurs on medium without plant growth regulators, although Tan *et al.* (2002) supplemented the basal medium of Lopez-Baez *et al.* (1993) with NAA, gibberellic acid (GA₃) and zeatin. Li *et al.* (1998) did not use a maturation medium, but distinguished two types of somatic embryos: (i) type I, a yellowish and translucent embryo with large cotyledons; and (ii) type II, a whitish embryo, with an opaque axis and cotyledons. Both types of somatic embryos were subcultured on regeneration medium until apparent maturity, when embryos with a fully developed embryonic axis or with an elongated hypocotyl radicle were present. At this stage, mature somatic embryos were transferred to a medium for conversion.

Lopez-Baez *et al.* (1993) utilized half-strength MS medium supplemented with 0.28 µM IAA, 0.25 µM IBA, 0.06 µM GA₃, 2.7 µM adenine sulphate and maltose (111 mM) for 4–5 weeks at low photon flux density (7 µmol/m²/s). Alemanno *et al.* (1997) compared somatic and zygotic embryo maturation, and described three distinct stages for the latter: (i) embryogenesis *sensu stricto*, defined by zygote division until the cotyledonary stage; (ii) growth, during which the embryo reached its final size; and (iii)

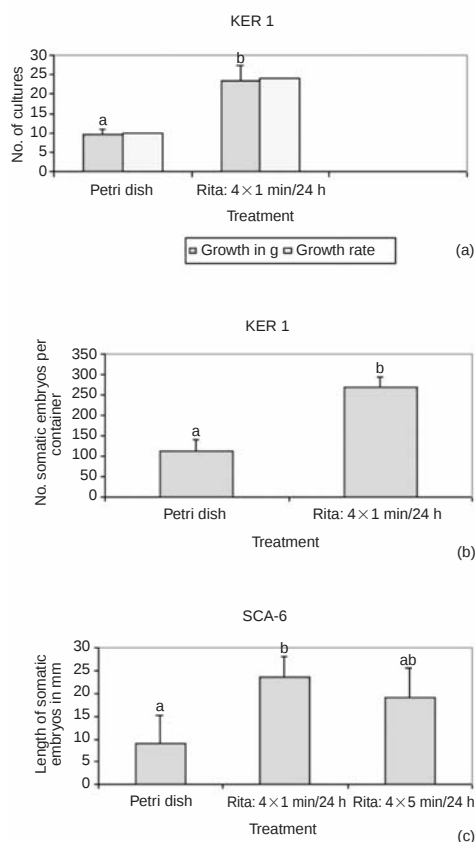


Fig. 21.1.2. Optimization of cocoa secondary somatic embryogenesis with the temporary immersion or RITA system. (a) Increased growth rate of the RITA system compared to semi-solid medium. (b) Increased growth rate corresponded to an increased number of somatic embryos. (c) Growth of somatic embryos was significantly increased by temporary immersion.

maturation, when reserve accumulation and desiccation occurred. Immature zygotic embryos had high levels of glucose, fructose and sucrose, and stachyose and raffinose increased during maturation; rhamnose, arabinose and galactose were newly synthesized. The Lopez-Baez *et al.* (1993) protocol was modified to include a growth phase on hormone-free medium for several weeks in darkness. Reserve starch synthesis accumulation was improved by replacing maltose with sucrose, and by the addition of abscisic acid (ABA) to stimulate protein synthesis. This caused a decrease in water content

(from 98% to 80%), but not as great as in zygotic embryos (30%). Soluble sugar composition of somatic embryos before maturation was similar to that of zygotic embryos, but sucrose, xylose and rhamnose concentrations were much higher while raffinose and stachyose contents were lower than those of zygotic embryos. The addition of 234 mM sucrose and 10 μ M ABA to maturation medium resulted in a significant increase of conversion and improved acclimatization.

Germination. Lopez-Baez *et al.* (1993) and Alemanno *et al.* (1997) stimulated germination and shoot development separately. Lopez-Baez *et al.* (1993) used a root elongation medium, containing NAA, GA₃, 2-isopentenyladenine (2iP), ABA and adenine sulphate with a 12 h photoperiod at a photon flux density of 60 μ mol/m²/s and 30–31°C during daytime and 25–26°C at night. The average root elongation rate of somatic embryos for seven genotypes after 4–5 weeks was 76.7%. Shoot development was obtained with the same medium without growth regulators and with activated charcoal. A conversion rate of 66.7% with seven genotypes was obtained. Alemanno *et al.* (1997) stimulated germination in the dark on medium containing GA₃, while shoot formation occurred in the light on a low-sucrose plant growth regulator-free medium. These conditions resulted in a germination rate of 0 to 64% for 'NA 79', and a shoot formation rate of 0 to 28%.

Li *et al.* (1998) defined a medium (plant regeneration (PR)) for root elongation and conversion, although root elongation could sometimes occur in regeneration medium (see above). PR medium contained a low amount of salts (DKW/5), glucose, sucrose, KNO₃ and no growth regulators, and cultures were incubated at 50 μ mol/m²/s with a 16 h photoperiod. During culture on PR medium, cotyledons of type I embryos expanded, while rapid tap root elongation occurred for type II somatic embryos. Type II somatic embryos produced shoots at a higher frequency than type I. Conversion rate for 'Scavina 6' somatic embryos was approx. 32% (Guiltinan and Maximova, 2001). L. Alemanno (unpublished data)

demonstrated the existence of a genetic component for conversion rate (Table 21.1.2). Lambert *et al.* (2002) modified the conversion medium defined by Li *et al.* (1998), using only glucose as a carbon source, and concluded that temperature was critical for conversion success, with small variations lowering the conversion rate. For efficient acclimatization, plantlets should already have at least three leaves and RH should be maintained at $> 80\%$. High rates of acclimatization have been obtained, e.g. 68.5% (Lopez-Baez *et al.*, 1993), 70% (Li *et al.*, 1998) and 95% (Lambert *et al.*, 2002).

The oldest field trial of somatic embryo-derived cacao plants was established in Ecuador with a mixture of four genotypes ('EET 48', 'EET 64', 'CC 260' and 'ICS 48'), with one group of 124 plants in June 1993 and a second group of 51 plants in August 1994 (Lopez-Baez *et al.*, 1997). Plants have shown normal growth and development, with formation of an orthotropic axis and leaves in a 3/8 phyllotaxy; normal jorqueting occurred at c. 15 months with three to five lateral branches. The trees flowered and produced fruits 18–24 months after planting. Three other research groups have field trials in progress. There are field trials on Saint Lucia in the Caribbean and in Brazil. Plant growth and development, including flowering and fruit set, are normal (M. Guiltinan, State College, USA, 2001, personal communication). Fontanel *et al.* (2002b) planted 110 plants produced in 1998 from 22 genotypes. A 3-year evaluation period detected normal architecture, flowering and fruiting. The production and quality of the seeds were identical to those of the grafted controls. More recently, these authors established larger field trials in three countries, each trial containing 1000 plants of three Ecuadorian genotypes (Fontanel *et al.*, 2002b). In Brazil, Mars Inc. planted between mid-1998 and 1999 more than 2000 plants derived from somatic embryos, mostly from witches' broom-resistant genotypes of various origins (TSH, LCTEEN, CCN, COCA), with normal plant development (S. Lambert, Ballarat, Australia, 2001, personal communication).

4.1.2. Protoplast isolation and culture

Thompson *et al.* (1987) first described isolation of protoplasts from cacao leaves. The protocol used 5–20 cm² of leaves during their expansion phase, incubated for 5 h at 25°C in 1% Pectolyase and 2% Cellulysin. Under these conditions, 4.5×10^6 protoplasts/g leaf tissue were isolated. Protoplasts were cultured in 0.6 M mannitol solution and after 40 h the viability of the protoplasts was 74%. More recently, Melo and Brar (1998) reported isolation and division of protoplasts from suspension cultures initiated from leaf callus, using a 24 h incubation time at 25°C with 0.5% Driselase, 1% Hemicellulase and 2% cellulase. The optimal mannitol concentration for protoplast division was 7 to 8% in a Czapeck solution. Further development has not been reported. Kanchanapoom and Kanchanapoom (1991) isolated protoplasts from suspension cultures initiated from epicotyl-derived callus. Cell walls were digested in a solution of 2% Driselase, 0.5 M sorbitol and 1 mM methyl ethane sulphonate (MES). Protoplast division was observed after 12–14 days. Somatic embryo-like structures were observed after 1 month of culture on MS medium supplemented with 7.4 μ M NAA.

4.1.3. Haploid plant recovery

The recovery of haploid plants from cultured anthers and microspores has not been reported; however, Dublin (1972, 1973) described the recovery of haploid ($n = x = 10$) cacao plants from naturally occurring polyembryonic seeds, and later Dublin (1978) described the production of dihaploid plants.

4.2. Genetic manipulation

4.2.1. Mutation induction and selection

The largely underexploited genetic diversity in *T. cacao* limits the potential application of mutation induction to obtain new variation, except for resistance to CSSV, for which a natural source of resistance may not exist. The level of resistance identified from genotypes

introduced from the Amazon is far from satisfactory, and mutation induction in cacao has been attempted to obtain resistance to CSSV (Adu-Ampomah *et al.*, 1996). Plants with resistance to CSSV were obtained from buds of three susceptible cultivars irradiated with 15–25 Gy, and selected at MV3 by grafting bark-patches of infected tissues, followed by inoculating with viruliferous mealybugs at MV4 and MV5 (Adu-Ampomah *et al.*, 1996). Attempts have also been initiated to induce mutation using embryogenic cultures (Adu-Ampomah *et al.*, 1988).

4.2.2. Genetic transformation

The lack of a reliable regeneration protocol from cell culture has delayed the development of a transformation system for elite cacao. Ferreira (2000) has attempted to genetically transform cupuassu.

Breeding objectives. The major objectives of cacao genetic transformation are resistance(s) to the main diseases and pests, especially when limited or natural sources of resistance are unknown. There is no obvious simple gene candidate for introduction into cacao to obtain resistance against any of the major pathogens, except for CSSV. Based on the current strategies for virus protection based on expression of viral coat protein or antisense or mutated transport protein, the development of CSSV-resistance is probably attainable.

There is an initiative in Brazil to sequence the complete genome of *C. pernicioso*, the cause of witches' broom disease (<http://www.lge.ibi.unicamp.br/vassoura/index.html>), and strategies to control this disease might derive from this programme. For pest resistance, work is under way to identify peptides against cocoa pod borer (Furtek *et al.*, 2001), and genes/strategies, such as *Bt* toxins, proteinases and α -amylase inhibitors and lectins, used in other crops, can be tested in cacao.

There are a few possibilities for manipulating yield components. Decrease of plant height (dwarfism), which is feasible with current technologies, would allow a higher tree density and facilitate disease control, i.e.

phytosanitary pruning for witches' broom. Increased pod set could be addressed by altering self-incompatibility, especially for the Upper Amazonian genotypes used as hybrid parents in most breeding programmes. Attempts to understand and identify gene products associated with self-incompatibility are under way (Ford and Wilkinson, 2002). There is potential for obtaining cultivars exhibiting pod abscission at maturity, a natural feature of cupuassu, by manipulating the formation of the abscission zone; this trait could reduce production costs significantly; however, this is a long-term objective. Expression of four genes associated with abscission zone formation (*jointless* abscission zone regulatory gene, cellulase, polygalacturonase and ethylene receptors) are being studied in cacao and cupuassu (Furtek *et al.*, 2001).

Manipulation of fat quality by altering enzymes in the fatty acid and triacylglycerol biosynthetic pathways is feasible. Changes in desaturation might allow cacao cultivation under lower growth temperatures without affecting cocoa butter hardness and quality. Yield and production costs of altered cocoa butter are probably not competitive with other plant production systems, e.g. canola and soybean. Manipulation of flavour quality by genetic transformation is unlikely for some time, because of lack of knowledge of the biochemical basis of flavour. Cocoa flavour development may be associated with the action of endoprotease and carboxypeptidase on seed storage proteins (vicilins) (Voigt *et al.*, 1994a,b), and sequences for some of these enzymes (aspartic proteinase *Tcap1* and *Tcap2*, and carboxypeptidase type III) have been obtained.

Accomplishments. Genetic transformation of cacao has been attempted using *Agrobacterium tumefaciens* and particle bombardment. Cacao is susceptible to wild strains of *A. tumefaciens* (Purdy and Dickstein, 1989), and cacao genotypes differ in their responses to wild virulent *A. tumefaciens* strains (Figueira *et al.*, 1997). *In vitro*-germinated plantlets have been inoculated with eight wild *A. tumefaciens* strains, differing for opine type, biovar and species of origin. All strains

induced tumours, but strains B653, 15.955, R10 and Ach5 caused larger galls at higher frequency. Disarmed *Agrobacterium* strains derived from these wild strains include LBA4404, a disarmed version of Ach5.

Transformed leaf callus has been recovered using *A. tumefaciens* A281 (pGPTV-Kan), but plants were not regenerated from the transformed cells (Sain *et al.*, 1994). Embryogenic cultures derived from petals and immature zygotic embryos were co-cultivated with LBA4404 (pBIN19::uidA), and transient expression of uidA (glucuronidase (GUS)) was observed under kanamycin selection (Figueira *et al.*, 1997). Maximum transient GUS expression was obtained when staminode and petal explants were co-cultivated with *Agrobacterium* LBA4404 (pTOK322) under vacuum 25 days after explanting (E. Mamani and A. Figueira, unpublished results). Embryogenic cultures derived from petals or staminodes co-cultivated with LBA4404 (pTOK322) did not exhibit any transient uidA expression, evaluated by histochemical assay 40–60 days after explanting (E. Mamani and A. Figueira, unpublished results). Regenerated transgenic cacao plants expressing green fluorescent protein (GFP) have been obtained by co-culturing floral parts with *A. tumefaciens* EHA105 (Maximova *et al.*, 2003). Perry *et al.* (2000) described transformation of embryogenic cacao cultures by particle bombardment; transient uidA activity was observed and no transgenic plants were obtained.

Because of the difficult transmission of CSSV to cacao and other hosts by its natural vectors or by mechanical wounding, a cloned DNA genome copy of CSSV was inserted into a binary vector (pBIN19) in *A. tumefaciens*, which was used to inoculate young cacao plants (Jacquot *et al.*, 1999). Viral DNA, CSSV coat protein and virions were detected and typical leaf symptoms and stem swellings were observed. Particle bombardment was also used to infect cacao seeds with CSSV, which were evaluated by symptom expression, enzyme-linked immunosorbent assay (ELISA) and dot-blot hybridization, and small bacilliform particles were observed by immunosorbent electron microscopy (Hagen *et al.*, 1994).

4.3. Cryopreservation

Cryopreservation of cacao tissues was first demonstrated by Pence (1991) using immature zygotic embryos. After slow hydrated freezing or fast desiccated freezing in liquid nitrogen, the embryos retained embryogenic competence. The optimum conditions involve preculture on medium containing 0.0876 M sucrose, then slow-freezing (0.4°C/min to –35°C) after a pre-treatment with 10% dimethyl sulphoxide (DMSO). Preculture of embryos on 0.35 M sucrose concentration increased survival rate of the explants, but decreased their embryogenic potential. Florin *et al.* (1998) cryopreserved embryogenic cultures, using a mixture of proembryonic masses (PEMs) and somatic embryos at different stages. The highest rate of survival (93%) was obtained following a pre-treatment of 22 days on semi-solid medium containing increasing sucrose concentrations (0.25 M, 0.50 M, 0.75 M and 1 M) followed by culture in liquid medium with 1 M sucrose, and a dehydration stage of at least 60 h after the sucrose treatment. Exposure of the explants to ABA before treatment enhanced their desiccation tolerance. Cryopreserved embryogenic cultures retained their ability to regenerate somatic embryos after freezing in liquid nitrogen, and *in vitro* plantlets were produced.

5. Conclusions

Biotechnology has been applied to cacao since the early 1970s; however, cacao has been difficult to manipulate *in vitro*. Somatic embryogenesis of cacao has application for germplasm distribution and cryopreservation. Lack of a reliable transformation system jeopardizes progress in applying genetic transformation to resolve breeding problems, e.g. resistance to CSSV. Molecular markers have been used intensively to study genetic diversity, in phylogenetic studies and more recently in genome mapping. There is great potential for application of genomic tools to accelerate cacao breeding; however, most research is conducted in consumer countries and the genetic stocks (populations, mutants, germplasm, related species) are only in

producing countries. The technologies are still quite expensive and require long-term commitment. International cooperation is required to support sustained progress in cacao improvement. Most importantly, small cacao growers should adopt and benefit from the new technologies to improve their living standards. Recently, there have been several international initiatives to coordinate worldwide cacao research, resulting in increased cooperation between teams in producer and consumer countries.

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22

Vitaceae

The *Vitaceae* consists of lianas, shrubs and occasionally herbs and contains 15 genera and approximately 700 species, which are widely distributed in the tropics and subtropics, but with representatives extending into the north and south temperate regions (Watson and Dallwitz, 1992 onwards). In addition to *Vitis*, common genera include

Ampelopsis and *Parthenocissus*. Most have a vining habit with tendrils opposite the leaves. Flowers are small, greenish, perfect or imperfect, with a calyptrate or valvate perianth, and the fruit is a berry. Plants are monoecious or dioecious. The genus *Vitis* contains the only edible species.

Reference

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22.1 *Vitis* spp. Grape

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1. Introduction

1.1. Botany and history

Vitis is divided into two subgenera, *Euvitis* Planch, the bunch grape species that all contain 38 somatic chromosomes ($2n = 2x = 38$) and *Muscadinia* Planch, the muscadine grapes with 40 somatic chromosomes ($2n = 2x = 40$) (Winkler *et al.*, 1974; Einset and Pratt, 1975). Species in each subgenus are interfertile but are only partially fertile between subgenera (Jelenkovic and Olmo, 1969). Estimates of the number of *Euvitis* species range from 28 to 43. These are separated into an American group of 18–28 species, an Asian group of 10–15 species and a European or central Asian group of one species (*V. vinifera*) (Einset and Pratt, 1975). *Muscadinia* contains three species; *V. munsoniana* and *V. rotundifolia* are native to the south-eastern USA and *V. popenoeii* Fennell is native to Central America. With the exception of muscadine cultivars, most cultivated grapes are either pure strains or hybrids of *V. vinifera*. Two wild subspecies of *V. vinifera*, *ssp. sylvestris* Gmel. and *ssp. caucasis* Vav., and one cultivated subspecies, *ssp. sativa* D.C., are recognized (Einset and Pratt, 1975).

Vitis contains climbing or trailing vines with alternate simple, serrate to sinuate, petiolate leaves. Inflorescences or tendrils (modi-

fied inflorescences) occur opposite the leaves. *Euvitis* tends to have shedding bark, whereas *Muscadinia* bark does not shed and contains conspicuous lenticels. Typically, there is a juvenile period, lasting for 1–3 years, during which time flowering occurs sporadically or not at all. In addition, leaf morphology is often distinct in juvenile vs. mature vines. Inflorescences are initiated in axillary buds during the summer that precedes bloom. Floral development accelerates in early spring of the following year. Paniculate flowers are small, with five stamens, a single pistil and five petals fused into a protective calyptra that is deciduous at anthesis (Radford *et al.*, 1968). Three types of flowers result in sex expression characteristic of certain species and cultivars. Perfect (hermaphroditic) flowers have functional stamens and pistils. Dioecious cultivars have female vines with pistillate flowers that contain functional pistils but reflexed stamens and sterile pollen, whereas male vines have staminate flowers with erect, functional stamens and aborted pistils (Einset and Pratt, 1975). Most *Vitis* species are dioecious, whereas most commercial varieties have been selected to have bisexual flowers. The inheritance of sex expression is poorly understood, but the bisexual trait can arise spontaneously in some species or can be transferred by hybridization. Pistil develop-

ment can also be stimulated in staminate flowers by application of synthetic and natural cytokinins. Anthesis usually occurs in mid to late spring during mid-morning. Fertilization is most likely by self-pollination in hermaphroditic flowers and by wind or insect pollination in dioecious vines (Einset and Pratt, 1975). Berries develop in clusters (bunches) of up to 100 or more in *Euvitis* or singly or in small clusters of three to five berries in *Muscadinia*. Each berry contains one to four seeds.

There is evidence of the use of *Vitis* dating back to 8000 BC in southern Europe. Cultivation of grape occurred no later than the Bronze Age (3000 BC) in the Mediterranean region (Zohary and Hopf, 1988). Currently, several *Vitis* spp. and their hybrids are in use (Einset and Pratt, 1975). *V. vinifera* L., the grape of antiquity, is native to southern Europe and the vicinity of the Black and Caspian Seas (Zohary and Spiegel-Roy, 1975). *V. vinifera* cultivars account for most of the world's production. Grape production has traditionally been associated with regions between 20 and 51° latitudes (Snyder, 1937). Although generally regarded as poorly suited to the tropics, adapted varieties are extending the range into more tropical and humid climates (Alleweldt and Possingham, 1988).

1.2. Importance

Grape is a nutritious source of natural sugars, vitamins and fibre. According to FAOSTAT (2004), grape production (60.9 Mt) is exceeded only by banana and citrus. Grape production is greater than any other temperate fruit crop; however, in value, grape surpasses all other fruit crops. The high value of grape stems from its widespread multiple uses for fresh fruit, juice, jelly, raisins and wine. The latter four products can be stored; this has promoted complex systems of trade and commercial exploitation which, in turn, have stimulated production. For example, raisin and wine futures are actively traded on major commodity exchanges. Especially for wine, a longstanding worldwide infrastructure is in place to promote marketing and

appreciation. Wine production represents the ultimate value-added use of a fruit crop, with costs sometimes exceeding thousands of dollars per bottle.

1.3. Breeding and genetics

Hybridization of grape is accomplished either by open or by hand pollination. For pistillate vines, controlled but open pollination can occur only if the pollen donor vine is also present. More commonly, however, emasculation followed by hand pollination is practised in order to produce controlled crosses. Pollen is either collected fresh from locally flowering vines or maintained for up to 4 years at -12°C and 28% relative humidity (RH) (Olmo, 1942b). Washed and dried seeds, collected from ripe berries, are dormant and must be cold-stratified at approximately 4°C for 60–90 days in moist sand prior to planting. Seed germination is typically poor but may vary from 0 to $> 90\%$ (Olmo, 1942a).

The long life cycle of grape in combination with barriers to hybridization that are imposed by dioecious vines has stimulated research designed to accelerate the normal flowering cycle as well as to change the sex of flowers. Srinivasan and Mullins (1978) showed that isolated tendrils (modified inflorescences) cultured *in vitro* developed into recognizable flowers when various cytokinins were applied directly. Later, they demonstrated this conversion *ex vitro* and obtained fertile flowers and fruit set (Srinivasan and Mullins, 1979, 1980a). Flowering and fruit set could be obtained with 3-month-old seedlings (Srinivasan and Mullins, 1981), demonstrating the potential utility of this technique for reducing the time for recurrent hybridization and selection. Cytokinin application to staminate flowers causes developing pistils that would otherwise be non-functional to become fertile. Such flowers can be pollinated and will set fruit (Negi and Olmo, 1966; Doazan and Cuellar, 1970; Moore, 1970). This technique potentially allows normally male vines to be utilized in breeding since they can then be crossed with other male or hermaphroditic vines.

Grape is very heterozygous and exhibits pronounced inbreeding depression (Winkler *et al.*, 1974; Einset and Pratt, 1975). Genotypes cannot be reproduced by seed, so outstanding selections must be propagated vegetatively. The juvenile period is relatively long, ranging from 1 to 6 years, depending on environmental conditions and management practices (Einset and Pratt, 1975). Thus, the fruit quality of progeny often cannot be assessed for several years. Resistance to certain diseases, e.g. Pierce's disease, cannot be confirmed until after several fruit-bearing years. Development of new cultivars by breeding and selection is therefore a long and tedious task. For example, introgressing specific resistance genes into an elite cultivar by hybridization is not easily accomplished, since the long juvenile period combined with heterozygosity and inbreeding depression make back-crossing and recurrent selection difficult or impossible (Alleweldt and Possingham, 1988). In most instances, only the F_1 generation can be selected. Therefore, conventional breeding can currently seek only to combine varieties in the hope that resulting hybrids will constitute an improvement over each parent. In practice, the hybrid typically possesses traits intermediate to each parent so that the fruit quality of one parent cannot usually be directly combined with the disease resistance of the other. The resulting hybrid most often possesses intermediate fruit quality and resistance, each of which is often at an unacceptably low level. In this instance, improvement to commercially acceptable levels is obtained by producing complex hybrids, resulting from selection and crosses with a new parent at each generation. This problem may be circumvented if the parents differ only for a single desirable trait, e.g. disease resistance. For example, if both parents have acceptable fruit quality, but only one has desirable disease resistance, the hybrid may have suitable fruit quality and resistance, resulting in a new and useful hybrid.

1.3.1. Rootstocks

Originally, most commercial plantings utilized ungrafted varieties; however, the

spread of certain pests, primarily nematodes and phylloxera (*Dactyloshaea vitifoliae*), has required grafting of fruit-bearing scions on to resistant rootstocks. Currently, most commercial production in Europe and California utilizes grafted vines. Rootstocks were first obtained directly by selection from nature, e.g. *V. champini* Planchon 'Dog Ridge' and *V. rupestris* Scheele 'St George'.

Major breeding objectives. Breeding objectives for rootstocks include development of pest and fungal resistance and tolerance of environmental conditions, e.g. lime-induced chlorosis, along with graft compatibility and transmission of high vigour and/or fruit yield to the scion (Winkler *et al.*, 1974).

Breeding accomplishments. Modern rootstocks are primarily interspecific hybrids between North American species (see Winkler *et al.*, 1974). In addition to resistance and tolerance characteristics mentioned above, breeding objectives include rootstocks that do not sucker when grafted. Rootstocks with resistance to Pierce's disease (*Xylella fastidiosa*), various fungi, nematodes and drought, e.g. 'Tampa' (Mortensen and Stover, 1982), and grape rootborer, e.g. 'Florilush' (Mortensen *et al.*, 1994b).

1.3.2. Scions

Major breeding objectives. Most major *V. vinifera* varieties grown today arose in prehistory as native races, as sports or by chance hybridization. Controlled hybridization has been utilized to produce improved cultivars (Einset and Pratt, 1975; Alleweldt and Possingham, 1988), particularly of seedless table grapes. Breeding objectives for grape include superior vigour, yield and quality; however, these objectives are difficult to achieve for grape because different characteristics are often needed for fruit, raisin, rootstock and wine cultivars. For a comprehensive treatment of grape breeding objectives, see Einset and Pratt (1975) and Alleweldt and Possingham (1988).

Disease and pest resistance. Grape is subject to bacterial, fungal, viral and

phytoplasma diseases and to nematodes (Bovey and Martelli, 1986; Pearson and Goheen, 1988). Insect pests serve as vectors for some pathogens, e.g. Pierce's disease bacteria, which is transmitted by members of the Cicadellidae (leafhoppers) known as sharpshooters, e.g. the glassy-winged sharpshooter (*Homalodisca coagulata*), and grape fanleaf virus, which is transmitted by nematodes. Some insect pests cause damage directly, e.g. grape root borer (*Vitacea polistiformis*) and phylloxera.

In conventional breeding approaches, existing sources of resistance are used, the overall objective being to combine disease resistance from one parent with quality aspects of another. Since *V. vinifera* is generally considered to have the most desirable fruit quality but is susceptible to many pests and diseases, including anthracnose (*Elsinoe ampelina*), black rot (*Guignardia bidwellii*), botrytis bunch rot (*Botrytis cinerea*), crown gall (*Agrobacterium tumefaciens*), downy mildew (*Plasmopara viticola*), eutypa dieback (*Eutypa lata*), various nematodes, phomopsis cane and leaf spot (*Phomopsis viticola*), phylloxera, Pierce's disease and powdery mildew (*Uncinula necator*), its hybridization with resistant species has been the only method available to produce resistant cultivars (Einset and Pratt, 1975; Galet and Morton, 1990).

Seedlessness. Development of improved seedless varieties by conventional breeding methods is complicated by the impossibility of using seedless vines as female parents (Gray *et al.*, 1987, 1990). Only the pollen parents can be seedless in such crosses and this restricts the number and types of desirable germplasm combinations that can be achieved, since the possibility of using seedless $\times$ seedless crosses or seedless vines as female parents is eliminated. Using seeded female parents, the frequency of resulting progeny that can be regarded as seedless (due to the presence of acceptably small and soft ovules at maturity) as well as the degree of seedlessness is low (Stout, 1936; Loomis and Weinberger, 1979). Two types of seedlessness are recognized in grape: (i) parthenocarpy, in which fruit development

does not require fertilization; and (ii) stenospermocarpy, in which fruit development requires fertilization but seedlessness occurs later when ovules cease normal growth and do not develop into seeds (see Soule, 1985, for definitions). Parthenocarpic cultivars can only be utilized as the pollen parent in breeding (Stout, 1936) and are of limited use in conventional breeding; whereas stenospermic varieties can be utilized as parents in seedless breeding if embryo rescue is used (Cain *et al.*, 1983; Emershad and Ramming, 1984; Spiegel-Roy *et al.*, 1985; Gray *et al.*, 1987, 1990).

Breeding accomplishments. Although there are hundreds of *V. vinifera* cultivars, genetic improvement has mostly been by clonal selection (Rantz, 1995). Clonal selection is used to maintain established cultivar standards, to determine the performance of clones in different environments and to identify sports with somatic mutations that might result in useful altered phenotypes. Several improved cultivars, including those with colour and seedlessness mutations, have been recovered using this approach. Commonly, clones with minor variations are selected for performance in specific geographic regions. Clonal selection has the advantage of utilizing otherwise desirable cultivars to recover improved phenotypes with only one or a few altered trait(s). On the other hand, clonal selection is unpredictable since it takes advantage of spontaneous mutation and one must be able to recognize and propagate an interesting sport whenever it may occur. In addition, some cultivars are more prone to somatic mutation than others (Rantz, 1995) and thus are more or less amenable to this method of genetic improvement. Due to the haphazard nature of clonal selection, other methods of genetic improvement that allow introduction of predictable genetic changes have been continually emphasized.

Disease and pest resistance. *V. labrusca* L. is native to eastern North America (Galet, 1988). Hybrids of *V. labruscana* $\times$ *V. vinifera*, some with additional native species parentage, are in widespread production in the

north-eastern USA (Cahoon, 1986), e.g. 'Concord'. Disease-resistant hybrids between *V. vinifera* and native American species are termed French-American hybrids and are used primarily for wine production (Hedrick, 1908; Winkler *et al.*, 1974; Einset and Pratt, 1975; Olmo, 1976). A number of other *Vitis* species have been used in breeding programmes to adapt high-quality *V. vinifera* germplasm to suboptimal environmental regions (Tarara and Hellman, 1990). In particular, *V. vinifera* has been hybridized with *V. aestivalis* ssp. *smalliana* and other species native to the south-eastern USA to form the so-called 'Florida hybrid bunch grapes', which are resistant to Pierce's disease (Halbrooks and Mortensen, 1989). *V. rotundifolia* Michaux and *V. munsoniana* Simpson, comprising the muscadine grape cultivars, are morphologically and genetically distinct from other *Vitis* species and are grown throughout the south-eastern USA.

Resistant species that have been successfully combined with *V. vinifera* to produce commercially useful hybrids include: (i) *V. aestivalis* Michx., which is resistant to downy mildew, powdery mildew and Pierce's disease; (ii) *V. berlandieri* Planch., which is resistant to black rot, downy mildew, powdery mildew, phylloxera and Pierce's disease; (iii) *V. candicans* Engelm., which is resistant to black rot, downy mildew, powdery mildew, Pierce's disease and root knot nematodes; (iv) *V. cinerea* Engelm., which is resistant to black rot, downy mildew and phylloxera; (v) *V. cordifolia* Michx., which is resistant to downy mildew and possibly phylloxera; (vi) *V. labrusca* L., which is resistant to downy and powdery mildew; (vii) *V. riparia* Michx., which is resistant to black rot, downy mildew, powdery mildew and phylloxera; (viii) *V. rupestris* Scheele, which is resistant to black rot, downy mildew, powdery mildew, phylloxera and Pierce's disease; and (ix) *V. rotundifolia* Michx., which is resistant to non-muscadine forms of black rot, downy mildew, powdery mildew, some nematodes and Pierce's disease (Galet and Morton, 1990). Work to introgress resistance genes from *V. rotundifolia* into *V. vinifera* is ongoing (Lu *et al.*, 2000), and 'Southern Home' is an

interspecific hybrid between *V. vinifera* and *V. rotundifolia* (Mortensen *et al.*, 1994a).

V. labrusca is most frequently utilized as a parent with *V. vinifera* due to its combination of cold tolerance, disease resistance and good fruit quality (Einset and Pratt, 1975). *V. vinifera* × *V. labrusca* hybrids are grown throughout eastern North America (Cahoon, 1986). *V. amurensis* Rupr. is another source of cold tolerance (Einset and Pratt, 1975). F₁ hybrids between *V. vinifera* and some of the species listed above possess other desirable adaptive characteristics in addition to disease resistance, but do not produce fruit of acceptable quality; thus, their use is limited to rootstocks. However, advanced generation hybrids with good fruit quality that contain one or more of these species crossed with *V. vinifera* are beginning to emerge as regionally adapted cultivars (Halbrooks and Mortensen, 1989).

Seedlessness. Embryo rescue allows progeny from seedless × seedless crosses to be obtained through ovule culture prior to developmental arrest (Cain *et al.*, 1983; Emershad and Ramming, 1984; Spiegel-Roy *et al.*, 1985; Gray *et al.*, 1987, 1990). Cain *et al.* (1983) reported that viable embryos could be rescued from eight of 14 self-pollinated seedless cultivars and from two seedless × seedless crosses. Plants were obtained from six of the self-pollinated varieties, but only those that had large abortive ovules. A single plant was obtained through culture of *V. vinifera* 'Thompson Seedless' (= 'Sultana', 'Sultanina') ovules, which are small and developmentally arrested, on filter paper bridges in liquid medium without growth regulators (Emershad and Ramming, 1984). Spiegel-Roy *et al.* (1985) obtained numerous plants from cultivars with small abortive ovules, using a semi-solid culture medium that contained indoleacetic acid (IAA) and gibberellic acid (GA₃). This study was confirmed by Gray *et al.* (1987). Developmental stage of the berry was found to be critical for successful embryo rescue. Ovules excised and cultured 40 to 60 days after pollination produced more embryos and plants than those cultured at earlier stages (Gray *et al.*, 1990; Gray and Hanger, 1993). Burger and

Trautmann (2000) showed that excision of the ovule and manner of placement on medium affected embryo development. Germination of the embryos and their growth into plants was enhanced with benzyladenine (BA) in the germination medium (Gray *et al.*, 1987, 1990).

The stage at which ovule development is arrested, relative to berry development, determines the degree of seedlessness. For example, cultivars in which ovule development becomes arrested at an early stage have very small, indistinct seed traces and are considered to be highly seedless, whereas those in which ovules develop further have more noticeable, sometimes objectionable, seed traces. Thus, the distinction between seededness and seedlessness is subjective. The genetic basis of stenospermocarpy was studied by Spiegel-Roy *et al.* (1990) using progeny of crosses between seeded females and seedless males. Approximately 30% of all progeny had fruit without noticeable seed traces and the overall distribution of seeded vs. seedless fruited progeny was approx. 3:1, suggesting that seedlessness is controlled by two complementary recessive genes.

Embryo rescue has been applied on a routine basis to the breeding of seedless grapes, including those with hybrid parentage (Goldy and Amborn, 1987; Gray *et al.*, 1990; Tsoлова, 1990). In practice, a higher percentage of progeny obtained by embryo rescue of seedless crosses are seedless and these exhibit a higher degree of seedlessness, i.e. smaller ovules, than those obtained by conventional breeding (Gray *et al.*, 1990). Embryo rescue could be used to introgress genes for seedlessness from *V. vinifera* into *V. rotundifolia* (Goldy and Amborn, 1987; Goldy *et al.*, 1988, 1989) and for breeding of early-maturing cultivars, whose berries typically ripen before their seeds are able to germinate (Ramming *et al.*, 1990). Embryo rescue has also been used for recovery of aneuploid plants from crosses among diploid, triploid and/or tetraploid parents (Park *et al.*, 1999). Such plants are trisomic and facilitate genetic analyses, but probably have no direct role in a breeding programme, since they are highly male and female sterile.

Seedless grape breeding has also been advanced by stimulating viable seed development in berries of a cultivar that normally has large but abortive ovules by applying growth regulators directly to grapevines (Ledbetter and Shonnard, 1990). This approach would constitute a cost-effective alternative to embryo rescue if it could be utilized in cultivars with small abortive ovules.

2. Molecular Genetics

2.1. Gene cloning

Genetic mapping of the grape genome utilizes specific DNA sequences as molecular markers in order to identify genes, promoters and other genetic elements, to distinguish cultivars from each other and to determine segregation of genetic traits during hybridization. Restriction fragment linked polymorphism (RFLP) technology is used to probe for polymorphisms and expressed sequence tags (ESTs) form the preliminary information base for identifying functional genetic elements.

Genetic diversity in grape species has been extensively studied using DNA markers. Microsatellite DNA sequences or simple sequence repeats (SSRs) mutate at a much higher rate and thus allow the rapid establishment of allelic diversity and DNA polymorphisms during the evolution of a species. They are ideal as genetic markers in conservation and population genetic studies (Cipriani *et al.*, 1994; Ellegren, 2000). In an analysis of > 300 grape cultivars, Bowers *et al.* (1999) demonstrated that 16 wine grapes grown in north-eastern France are probably derived from a single pair of parents, 'Pinot' and 'Gouais Blanc', based on 32 microsatellite loci. Using SSR markers, 40 grape accessions in the US Department of Agriculture (USDA) collection have been differentiated into three groups: (i) a group of nine Middle Eastern cultivars; (ii) a group of 22 from Russia and Afghanistan that are morphologically similar to 'Thompson Seedless'; and (iii) a group of 11 mostly eastern European wine cultivars (Dangi *et al.*, 2001). The

genetic diversity of clones within a cultivar has also been studied using amplified fragment length polymorphism (AFLP) analysis (Scott *et al.*, 2000). Over 3000 markers in grape have been generated from 64 AFLP primer combinations. Among these markers, two have been determined to be associated with the unique earlier bud burst characteristics found in a somatic mutant from 'Flame Seedless' (Scott *et al.*, 2000).

Genetic mapping in grape was initiated in the early 1990s. Striem *et al.* (1990) first reported the differentiation of seven genetically unrelated *V. vinifera* genotypes by using two different microsatellite DNA probes. Tschammer and Zyprian (1994) used RFLP analysis to characterize related Riesling and Burgundy cultivars. Concerted efforts worldwide have led to the description of genetic linkage maps of grape species, and these have been utilized to identify marker-associated agronomic traits, including quantitative trait loci (QTLs). Lodhi *et al.* (1995) used 422 random amplified polymorphic DNA (RAPD), 16 RFLP and isozyme markers to construct a genetic linkage map based on an interspecific hybrid grape population of 'Cayuga White' × 'Aurore'. In their study, up to 22 linkage groups with an average distance of 6.1 cM between markers covering 1477 cM of the grape genome were established. In later studies, Dalbo *et al.* (2000) demonstrated the correlation of a single locus controlling sex in grapes with a microsatellite marker in genetic linkage maps constructed using an interspecific hybrid population of the cross 'Horizon' × 'Illinois 547-1' with 318 DNA markers. In addition, improved selection of the QTL for powdery mildew resistance in segregating populations was achieved using molecular markers and the established linkage maps (Dalbo *et al.*, 2001).

In order to facilitate gene expression analysis in grape, several projects have been initiated to sequence a large number of ESTs derived from various complementary DNA (cDNA) libraries. Ablett *et al.* (2000) analysed c. 5000 ESTs associated with berry and leaf tissues of *V. vinifera*. Among these ESTs, only 1% matched known *Vitis* proteins, while the majority of the remaining ESTs showed

sequence homology with known plant proteins and non-plant proteins. Ablett *et al.* (2002) reported the sequencing of up to 40,000 ESTs covering 12 cDNA libraries constructed from leaf, root, flower, berry, bud and callus of several grape genotypes. Analysis of these ESTs revealed 17,588 distinct genes with a wide range of functional categories. Most of the characterized gene sequences in grape can be accessed via web-based databases including GenBank and <http://www.scu.edu.au/research/cpcg/genomics/index.htm>.

Grape bacterial artificial chromosome (BAC) libraries containing genomic DNA inserts up to 355 kb in size have been reported (Chalhoub *et al.*, 2002; Tomkins *et al.*, 2002), and will benefit genome organization studies and gene cloning in grapes.

2.2. Genetic markers

Grape taxonomy is difficult and controversial, since many conventional criteria for defining species, e.g. chromosome number, cross-fertility and geographic distribution, are not useful within subgenera and much natural cross-hybridization occurs between species (Olmo, 1976; Rogers and Mortensen, 1979; Cahoon, 1986; Halbrooks and Mortensen, 1989; Galet and Morton, 1990). Cultivar identification generally involves comparisons of vine and berry morphology, characters that are often neither distinct nor consistent in expression (Parfitt and Arulsekar, 1989). As a result, molecular markers have been utilized to determine taxonomic affinities, as an aid to speciation and to serve as reliable indicators for identification of specific cultivars.

Protein markers are preferable to morphological markers, because they represent genetic traits that occur independently of environmental influences. Electrophoretic separation of isozymes has been shown to follow genetic segregation patterns. Inheritance studies have shown that isozymes of a given enzyme in grape are usually controlled by a single locus, although multi-locus control can occur (Loukas *et al.*, 1983; Weeden *et al.*, 1988). This

is a convenient and accurate method for determining taxonomic affinities and cultivar identification, e.g. isozyme banding patterns for acid phosphatase (ACPH), peptidase (PEP) and glucose-6-phosphate isomerase (GPI) differ in 27 of 29 distinct grape species and cultivars (Subden *et al.*, 1987). Phosphoglucomutase (PGM) and GPI have been used to separate 145 *V. vinifera* and *Vitis* spp. cultivars into 52 distinct groups (Parfitt and Arulsekhar, 1989). Similarly, the isozyme band for ACPH, catechol oxidase (CO), indophenol oxidase (IPO) and leucine amino peptidase (LAP) can be used to distinguish > 60 *V. vinifera* cultivars (Wolfe, 1976). Use of tissue derived from actively growing shoot tips decreased problems with isozyme resolution caused by high tannin concentrations (Walters *et al.*, 1989). For optimum isozyme resolution, shoot and callus tissue collected at the end of winter is reportedly better than shoot tissue collected in spring (Kozma *et al.*, 1990).

Analysis of isozyme banding patterns has shown practical applications in several problems related to grape genetics and reproductive biology. In taxonomy, GPI and PGM banding patterns have shown that two long-standing, geographically separated populations of *V. vinifera* 'Cabernet Franc' are actually distinct, and one population is apparently *V. vinifera* 'Carmenere' after additional morphometric analysis (Calo *et al.*, 1990). Two closely related patented cultivars have been shown to be different from each other by comparing isozyme banding patterns (Striem *et al.*, 1990). As examples of breeding applications, the parentage of plants from ovule culture has been studied using GPI to show that 67% of progeny recovered from controlled pollinations are clearly of hybrid origin; 21% had band patterns that could have arisen by either selfing or crossing and 12% were not hybrids, probably occurring by selfing (Gray *et al.*, 1990). Isocitrate dehydrogenase (IDH), GPI, malate dehydrogenase (MDH) and PGM have been used to distinguish hybrids between *V. rotundifolia* and *V. vinifera* from selfs (Chaparro *et al.*, 1989). Both GPI and IDH have been used to investigate the origin of polyembryos

obtained during embryo rescue of progeny from controlled pollinations between seedless cultivars (Durham *et al.*, 1989). Analysis of 11 progeny from five polyembryonic ovules demonstrated that polyembryos originated through several distinct mechanisms: (i) fertilization and development into embryos of more than one cell in the embryo sac; (ii) somatic embryogenesis from the zygote; and (iii) embryogenic development from unfertilized gametic cells and the zygote. This latter demonstrates the usefulness of isozyme analysis for studying basic aspects of plant reproductive biology. Isozymes have been used to study potential genetic variation in somatic tissue. Differences between micropropagated plants and source plants were determined by quantitatively comparing ACPH, GPI and PGM banding patterns (Botta *et al.*, 1990). Micropropagated and source plants of each cultivar were shown to have identical banding patterns but differed in the amount of isozyme present. The amount of ACPH was higher in source plants, whereas the relative amounts of GPI and PGM were lower.

3. Micropropagation

Micropropagation of grapevine is well established for a wide range of grape species (Chee and Pool, 1983; Gray and Fischer, 1985; Gray and Klein, 1988, 1989; Gray and Benton, 1991). The technique can be used for rapidly increasing newly developed cultivars when propagation stock is limited. Endophytic microorganisms can be eliminated during culture initiation and sanitation can be maintained.

Shoot tips and nodes from rapidly growing vines are utilized as explants on semi-solid Murashige and Skoog (1962) (MS) medium with BA. Culture proliferation and maintenance are simple, since nodal tissue from cultures can be used for subculture (Fig. 22.1.1). Shoots obtained can be rooted on semi-solid medium without BA, but supplemented with IAA, naphthaleneacetic acid (NAA) or indolebutyric acid (IBA). Rooted shoots are acclimatized to ambient conditions and are established first as potted



Fig. 22.1.1. Shoot tip/nodal culture of grapevine, demonstrating shoot proliferation on medium containing a cytokinin, such as benzyladenine.

plants before grafting and/or establishment in the field.

Skene *et al.* (1988) showed that the ploidy of micropropagated plants is stable after medium-term *in vitro* storage; however, plants of *V. vinifera* 'Albarino' established in field trials had modified leaf morphology, which was regarded as reversion to a juvenile trait. The plants also had low fertility and poor yield (Martinez and Mantilla, 1995). A 10-year study of micropropagated *V. vinifera* 'Chardonnay' and 'Pinot Noir' showed that juvenile characteristics had disappeared within 8 years (Deloire *et al.*, 1995).

4. Elimination of Pathogens

The utility of micropropagation for eliminating endophytic bacteria, viruses and viroids has been demonstrated in several studies (Barlass *et al.*, 1982; Duran-Vila *et al.*, 1988; Robacker and Chang, 1993). Gifford and Hewitt (1961) demonstrated that a combination of thermotherapy and *in vitro* culture could be used to eliminate fanleaf virus. Leonhardt *et al.* (1998) used thermotherapy

and shoot culture to eliminate several viruses from several cultivars, with confirmatory indexing under way. Duran-Vila *et al.* (1988) cultured 0.1–0.2 mm shoot tips from existing *in vitro* cultures which were infected with two viroids. They reported all cultured shoot tips of *V. vinifera* 'Cabernet Sauvignon' survived and were free of viroids. Robacker and Chang (1993) showed that *in vitro* culture effectively eliminated *X. fastidiosa*, the causal agent of Pierce's disease, from muscadine grapevine.

5. Somatic Cell Genetics

5.1. Regeneration

Although embryogenic and organogenic regeneration pathways have been described for grape, the former has been the subject of most investigation.

5.1.1. Somatic embryogenesis

Two early reports concerning successful plant regeneration in *Vitis* (Hirabayashi *et*

al., 1976; Favre, 1977) did not mention somatic embryogenesis, but photographs and descriptions, in retrospect, suggested it. Somatic embryogenesis was first unequivocally reported by Mullins and Srinivasan (1976), who cultured unfertilized ovules of *V. vinifera* 'Cabernet Sauvignon' in liquid medium that contained naphthoxyacetic acid (NOA) and BA. Proliferating nucellus-derived embryogenic cultures were extruded through the micropyle and somatic embryos developed from these cultures. Somatic embryos germinated following transfer to semi-solid medium that contained GA₃ and 2-isopentenyladenine (2iP). Long-term maintenance of cultures was not described. Krul and Worley (1977) induced embryogenic cultures from various leaf, petiole and stem segments of the French-American hybrid 'Seyval' on semi-solid medium that contained 2,4-dichlorophenoxyacetic acid (2,4-D). Somatic embryos developed after transfer of cultures to medium containing NAA and BA, and germination occurred on medium without growth regulators. The original embryogenic line could be maintained indefinitely through secondary embryogenesis on growth regulator-free medium. A US patent describing this method of somatic embryogenesis and plant production was awarded (Krul, 1985). The current interpretation of somatic embryogenesis has been the result of several research groups (Table 22.1.1).

Induction. Induction of embryogenic cultures is genotype-dependent and, increasingly, more cultivars are being successfully introduced into culture (Table 22.1.1). Most reports involve *V. vinifera* or its hybrids, e.g. 'Seyval Blanc', 'Villard Blanc' and 'Villard Noir'. Somatic embryogenesis has been reported for very few *V. vinifera* cultivars, although this problem is constantly being addressed.

The source of explant for initiating cultures is critical. Floral tissues, particularly anthers or ovules, have been widely used; however, leaves, shoot tips, tendrils and zygotic embryos have also been utilized (Table 22.1.1). Anthers are the explant of choice for inducing embryogenic cultures.

Recent reports suggesting improved embryogenesis from leaves (Harst, 1995; Torregrosa *et al.*, 1995) have involved a limited number of cultivars, e.g. 'Seyval Blanc', that are known to be highly embryogenic (Krul and Worley, 1977; Robacker, 1993). Explant developmental stage as well as preconditioning can be important. Anthers approx. 0.5 mm long have been optimal for inducing embryogenic cultures of *V. vinifera* 'Cabernet Sauvignon' (Mauro *et al.*, 1986) and leaves 1.5–5.0 mm long produced embryogenic cultures for several cultivars (Stamp and Meredith, 1988a). Chilling anthers at 4°C for 72 h prior to culturing increases induction (Rajasekaran and Mullins, 1979).

Sex of the stock plant can affect induction. Rajasekaran and Mullins (1983b) noted that induction frequency of embryogenic cultures from anthers of male vines was greater than that of hermaphroditic vines and that anthers from female vines did not form somatic embryos at all. Treatment of a male vine with cytokinin to feminize the flowers inhibited anthers while it stimulated the ovules to produce somatic embryos (Srinivasan and Mullins, 1980b; Rajasekaran and Mullins, 1983b). Gray and Mortensen (1987) observed that anthers and ovaries of untreated female *V. longii* 'Microsperma' are embryogenic. Therefore, the importance of sex expression for induction is not clear. Growth responses of tissues from male and female vines can clearly be manipulated with growth regulators.

There is currently no consensus regarding basic embryogenic culture medium formulations for grape. Most reports involve modified MS medium and Nitsch's medium (Nitsch and Nitsch, 1969; Table 22.1.1). Mullins and co-workers (Mullins and Srinivasan, 1976; Rajasekaran and Mullins, 1979; Mullins and Rajasekaran, 1980) utilized liquid medium, whereas others have used semi-solid medium. Stamp and Meredith (1988a) compared liquid and semi-solid media for leaf culture and found only the latter was suitable. However, Jayasankar *et al.* (1999a) achieved high-frequency embryogenesis and plant regeneration in a liquid medium composed of B5 major salts

Table 22.1.1. Chronology of selected literature concerning somatic embryogenesis in grape.

Date	Species/variety	Explant ^a	Medium ^b	Plant growth regulators used ^c	Culture age ^d	Germination treatment	References
1976	<i>V. vinifera</i> 'Cabernet Sauvignon'	O-Nu	N	5-20 NOA 5-10 BA	NG	1 GA + 5 IP	Mullins and Srinivasan
1977	<i>Vitis</i> hybrid 'Seyval Blanc'	S, L, F	MS	4.5 2,4-D 0.4 BA	6 mo.	none	Krul and Worley
1979	<i>V. vinifera</i> × <i>V. rupestris</i>	A	N	5 2,4-D 1 BA	NG	4C	Rajasekaran and Mullins
1980	<i>V. longii</i>	A	N	5 2,4-D 1 BA	NG	4C	Mullins and Rajasekaran
	<i>V. rupestris</i>						
	<i>V. vinifera</i> 'Grenache'						
	<i>V. vinifera</i> × <i>V. rupestris</i>						
1980	<i>V. vinifera</i> 'Cabernet Sauvignon'	O-Nu	N	5 NOA or 5 2,4-D then 10 NOA 1 BA	NG	Plants not obtained	Srinivasan and Mullins (b)
	'Grenache'						
1982	<i>V. vinifera</i> × <i>V. rupestris</i>						
	<i>Vitis</i> hybrid 'Seyval Blanc'	NG	NG	NG	Indefinite	1 BA	Krul
1983	<i>V. longii</i>	A	N	5 2,4-D 1 BA	NG	NG	Rajasekaran and Mullins (a,b)
	<i>V. rupestris</i>						
	<i>V. vinifera</i> 'Grenache'						
	'Sumoli' × 'Cabernet Sauvignon'						
	<i>V. vinifera</i> × <i>V. rupestris</i>						
	<i>Vitis</i> hybrid 'Villard Noir'						
	'Villard Blanc'						
	M.G. 60-44						
1986	<i>V. vinifera</i> 'Cabernet Sauvignon'	A	MS	4.5 2,4-D then 0.5 NAA 1 BA	NG	4C + cotyledon removal	Mauro et al.
1987	<i>V. longii</i>	A & Ov	MS	5 2,4-D 1 BA	20 mo.	1 BA or dehydration	Gray(b); Gray and Mortensen Stamp
1988	<i>V. rupestris</i>	L	N	4.5 NOA 0-4.4 BA	NG	none or various growth regulators	and Meredith(a)
	<i>V. vinifera</i>						"
1988	<i>V. rupestris</i>	A	N	4.5 2,4-D or 4.5-7 NOA 0.9-4.4 BA	"	"	"
	<i>V. vinifera</i> 'Cabernet Sauvignon'						
	'Cardinal'						
	'Grenache'						
	'Sauvignon Blanc'						
	'Thompson Seedless'						
	'White Riesling'						
	<i>V. vinifera</i> × <i>V. rupestris</i>						

	Z	N	4.5 2,4-D or 0-4.4 BA	12 mo.	"	Stamp and Meredith(b)
1988	<i>V. longii</i> <i>V. vinifera</i> 'Chardonnay' 'French Colombar' 'Grenache' 'White Riesling'					
1989	<i>V. longii</i> <i>V. vinifera</i> 'Thompson Seedless' <i>Vitis</i> hybrids	A, Ov, L MS "	5 2,4-D 1 BA 10 IAA 1 GA 1 BA	48 mo. 42 mo.	1 BA or dehydration 1 BA or dehydration none	Gray Gray Matsuta and Hirabayashi
1989	<i>V. vinifera</i> 'Koshusanjaku'	L N, MS	5-10 2,4-D w./wo. transfer to 1 2,4-D 5-10 BA or 5-10 TDZ or 5-10 KT	24 mo.		
1992	<i>V. rotundifolia</i> 'Dixie', 'Fry', 'Nesbitt', 'Welder'	Z N	1 mg/l 2,4-D, 0.2 mg/l BA	12 mo.	1 BA	Gray
1993	<i>V. rupestris</i>	LP				Martinelli et al.
1993	<i>V. rotundifolia</i> 'Fry', 'Regale'	L N	9 2,4-D, 4.4 BA	NG	1 BA	Rbacker
1994	<i>V. riparia</i>	A MS	1.1 mg/l 2,4-D 0.2 mg/l BA	NG	MS liq	Mozar and Sule
1995	<i>V. vinifera</i> 'Seval Blanc', <i>V. thunbergii</i> , <i>Vitis</i> hybrid 'Chancellor'	L N	4 mg/l NOA 0.9 mg/l TDZ	24 mo.	none	Harst
1995	<i>V. vinifera</i> 'Centennial', 'Novomuscat', 'Ruby Seedless', 'Superior Seedless'	A MS	2 mg/l 2,4-D 0.2 mg/l BA 5 mg/l IASP 1 mg/l ABA	18 mo.	0.1 NAA	Perl et al.
1995	<i>Vitis</i> hybrid 'Seval Blanc'	L P N	4 mg/l NOA 0.9 mg/l TDZ	NG	none	Reustle et al.
1995	<i>V. vinifera</i> × <i>V. rotundifolia</i>	L MS	5 2,4-D 1.1 BA then 1.2 2,4-D	24 mo.	5 IAA 1.1 BA then 1.1 BA	Torregrosa et al.
1996	<i>V. vinifera</i> 'Mission'	A MS	4.9 2,4-D 4.4 BA	NG	1.1 IAA or 1 IBA and 6.6 BA	Popescu
1997	<i>V. vinifera</i> 'Koshusanjaku'	L P N	1 2,4-D then 2 mg/l NAA 0.5 mg/l BA	NG	none	Zhu et al.
1997	<i>V. vinifera</i> 'Thompson Seedless' 'Sonaka', 'Tas-e-Ganesh'	T N	10 NAA 0.4 GA 1.82 mM BA then 1 BA	NG	NG	Salunkhe et al.
1998	<i>V. vinifera</i> 'Denuda', 'Portan', 'Syrah', 'Ugni Blanc'	A MS	5 2,4-D	24 mo.	none	Torregrosa
1999	<i>Vitis latifolia</i> L.	A N	20 2,4-D 9 BA	NG	none	Salunkhe et al.

Continued

Table 22.1.1. Continued.

Date	Species/variety	Explant ^a	Medium ^b	Plant growth regulators used ^c	Culture age ^d	Germination treatment	References
1999	<i>V. vinifera</i> 'Chardonnay', 'Thompson Seedless'	A, O, L	MS then B5 liq	5 2,4-D 1 BA then 4.5 2,4-D	NG	none	Jayasankar et al.
1999	<i>Vitis</i> hybrid 'Seyve Villard 5276'	L	N	4 mg/l NOA 0.9 mg/l TDZ	NG	none	Passos et al.
2001	<i>V. vinifera</i> 'Cabernet Sauvignon', 'Chardonnay', 'Chenin Blanc', 'Muscat Gordo Blanco', 'Pinot Noir', 'Riesling', 'Sauvignon Blanc', 'Semillon', 'Shiraz'	A	MS or N	various combinations & concentrations of 2,4-D, NOA, KT, BA, TDZ	NG	none	Iocco et al.
2001	<i>Vitis</i> × <i>Labruscana</i> 'Fredonia', 'Niagara'	O	N	17 IASP 9 2,4-D 1 BA	24 mo.	0.5 NAA 0.4 BA	Motoike et al.
2001	<i>V. rupestris</i>	PE	MS liq	0.1 mg/l IBA	120 mo.	none	Martinelli et al.

^aA = anthers; F = floral tissue; L = leaves; NG = explant not given; O = fertilized ovule; O-Nu = unfertilized ovule cultured – nucellus confirmed to be source of embryogenic cells; Ov = unfertilized ovaries; P = protoplast; PE = petiole; S = shoot tip; Z = zygotic embryos.
^bMedia formulae are usually modified versions of the following: Please consult each reference for details. B5 = Gamborg et al. (1968); MS = Murashige and Skoog (1962); N = Nitsch and Nitsch (1969); liq = liquid suspension culture medium.
^cPlant growth regulators are expressed in micromolar concentrations, unless otherwise noted. Auxins: 2,4-D = 2,4-dichlorophenoxyacetic acid; IAA = indole-3-acetic acid; IASP = indole-3-acetyl-L-aspartic acid; NAA = naphthaleneacetic acid; NOA = naphthoxyacetic acid. Cytokinins: BA = 6-benzyladenine; KT = *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea; TDZ = thidiazuron = *N*-(1,2,3-thiadiazol-5-yl)-*N'*-phenylurea. Absciscic acid: ABA; gibberellic acid: GA.
^dCulture age = Time period over which embryogenic cultures could be maintained.

(Gamborg *et al.*, 1968) and MS medium minor salts. Other medium addenda include 20–60 g/l sucrose, 1 mg/l adenine, 100 mg/l glutamine and 10 mg/l phenylalanine (Mauro *et al.*, 1986).

Generally, the phenoxy-auxins, 2,4-D or NOA, in combination with BA, are utilized for induction of embryogenic cultures; however, IAA combined with GA₃ has been used to induce embryogenic cultures from fertilized ovules (Gray, 1989). Thidiazuron (TDZ) and *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (KT-30) are superior to BA (Matsuta and Hirabayashi, 1989).

The origin of embryogenic cells in primary explants of grape is obscure. Rajasekaran and Mullins (1983a) suggested that anther somatic tissues and not gametic cells gave rise to embryogenic cultures, since haploids and homozygous diploids were not among regenerants. Somatic embryogenesis from anther cultures involves the filament where it connects to the anther sacs (D.J. Gray, unpublished). Embryogenic cultures are induced from the nucellus in unfertilized ovules (Mullins and Srinivasan, 1976), whereas most polyembryos from fertilized ovules are of zygotic embryo origin (Durham *et al.*, 1989). In cultured leaves, embryogenic cultures arise from mid-vein and adjacent lamina tissue (Stamp and Meredith, 1988a). Embryogenic cell cultures are composed of small, starch- and lipid-rich cells, interspersed with larger vacuolate cells (Gray and Mortensen, 1987). Embryogenic cells are easily identified by their prominent nuclei and nucleoli as well as densely stained cell walls and cytoplasm (Krul and Worley, 1977). Somatic embryos appear to develop from single embryogenic cells with development of a suspensor (Krul and Worley, 1977; Gray and Mortensen, 1987; Jayasankar *et al.*, 2001a).

Genotype is probably the critical factor affecting induction of embryogenic cultures. Induction of embryogenic cultures from leaves of several major *V. vinifera* cultivars (Stamp and Meredith, 1988a) has been difficult to repeat. Environmental factors can have a more significant effect on induction than medium and culture conditions and possibly genotype. This could explain the

lack of repeatability of published studies and the disagreement among different laboratories with respect to optimal culture conditions.

Maintenance. Maintenance of embryogenic cultures is necessary in order to apply many biotechnological procedures. Mullins and co-workers (Table 22.1.1) suggested that somatic embryogenesis is a terminal event and that the cultures could not be maintained; however, Krul (1985) showed that cultures could be maintained for at least 6 months and possibly indefinitely. Time periods for culture maintenance have been 12 or more months (Table 22.1.1). Perl *et al.* (1995) maintained embryogenic cultures of several seedless *V. vinifera* cultivars for 18 months with abscisic acid (ABA) in the medium. Torregrosa (1998) maintained embryogenic cultures of several *V. vinifera* cultivars for 2 years on medium containing 2,4-D. Motoike *et al.* (2001) maintained embryogenic cultures of two *Vitis* × *labruscana* cultivars for 2 years. We routinely maintain embryogenic cultures of several cultivars for 4 or more years (D.J. Gray, unpublished). Embryogenic cultures have been maintained either by continual transfer on to medium containing auxin with or without cytokinins or by subculture on semi-solid medium without growth regulators (Fig. 22.1.2). Gray and Mortensen (1987) noted that embryogenic cultures maintained with auxin continually separated into embryogenic and non-embryogenic sectors, whereas Stamp and Meredith (1988a) and Matsuta and Hirabayashi (1989) did not report this. The latter reported that embryogenic cultures could be maintained by omitting vitamins, glycine and inositol from the medium. Maintenance of cultures on growth regulator-free medium was reported by Krul and Worley (1977), Krul and Mowbray (1984) and Gray and Mortensen (1987). Under these conditions, secondary embryos develop from previously formed somatic embryos, usually at the hypocotyl–radical boundary of subtending embryos.

It is unclear whether prolonged culture of embryogenic cells will result in mutation or other genetic abnormalities in grape as has been noted for some crops (Hammerschlag,

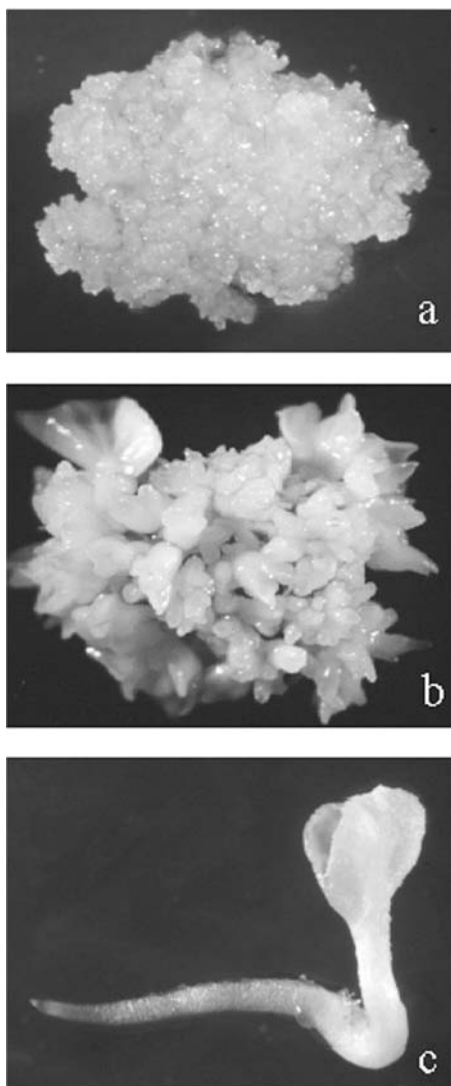


Fig. 22.1.2. Somatic embryogenesis in grapevine. Induction of embryogenic cultures (proembryonal masses) and maturation and germination of somatic embryos.

1992). Martinelli *et al.* (2001) demonstrated plant regeneration from 10-year-old embryogenic cultures of *V. rupestris*. A cell line of *V. longii* has been in continuous culture for 18 years; however, these cultures have lost their embryogenic competence (D.J. Gray, unpublished). Epigenetic changes and/or mutations almost certainly accumulate, which negatively affects the competence of embryogenic cultures.

Development and maturation. Sucrose concentration affects embryo development and maturation (Compton and Gray, 1996). Cultures maintained on semi-solid medium with 90 or 120 g/l sucrose produced more cotyledonary stage somatic embryos than those maintained on lower concentrations. Cultures transferred to medium with 150 g/l sucrose produce a higher frequency of somatic embryos capable of plant development. Starch is not present in appreciable amounts in either suspensors or somatic embryos, although lipids are abundant (Gray and Mortensen, 1987). Somatic embryos that develop on semi-solid medium tend to have smaller suspensors and more enlarged cotyledons than those that develop from suspension cultures (Jayasankar *et al.*, 1999a), which have elongated suspensors and small cotyledons (Jayasankar *et al.*, 2001a, 2003a). Such somatic embryos also differ from each other physiologically in that the former type is dormant.

Somatic embryos pass through developmental stages, possess normal epidermal, cortical and vascular tissues and tend to be opaque-white from the early torpedo stage to germination (Gray, 1988, 1995; Fig. 22.1.2). Somatic embryos that are anatomically and morphologically similar to all zygotic embryo stages are commonplace; however, a number of developmental abnormalities can occur. The most common abnormality of somatic embryos on semi-solid medium is the development of more than two, often fused, cotyledons or somatic embryos that are larger than zygotic embryos (Gray and Mortensen, 1987). With liquid medium-derived somatic embryos, the cotyledons are greatly reduced in size and smaller than those of zygotic embryos (Jayasankar *et al.*, 1999a). Jayasankar *et al.* (2002) described a unique monocotyledonous morphotype of grape that occurred in liquid medium-derived somatic embryos. The monocotyledonous somatic embryos germinated and developed into plants more rapidly than dicotyledonous somatic embryos. The increased rate of development was attributed to the presence of a larger apical meristem, compared to the dicotyledonous embryos. Monocotyledonous and dicotyle-

donous somatic embryos had the same rates of survival and plant development.

Germination and plant development.

Although plant recovery from grape somatic embryos was described by Mullins and Srinivasan (1976), specific germination requirements have varied widely. Somatic embryos have been subjected to cold stratification, auxins, cytokinins, GA₃ or nothing at all to induce germination and recover plants (Table 22.1.1; Fig. 22.1.2). Somatic embryos that develop on growth regulator-free medium always require a pre-treatment to germinate (Krul, 1985; Gray, 1987c, 1989; Gray and Mortensen, 1987). Grape seed exhibits a type of dormancy that is alleviated by cold stratification. Germination occurs after approximately 4 weeks at 4°C (Flemion, 1937). Grape somatic embryos resemble zygotic embryos, and germination is improved by cold treatment, providing evidence that they are also dormant (Gray and Mortensen, 1987). Where no pre-treatment has been required for grape somatic embryo germination, it has occurred when auxins with or without cytokinins have been used to produce somatic embryos (Table 22.1.1). These somatic embryos were subjected to a dormancy-breaking pre-treatment.

The grape embryogenic culture system is perhaps the best example of somatic embryo dormancy. Grape somatic embryos become well developed and are morphologically similar to zygotic embryos, but germinate poorly, suggesting that the block to germination is at the physiological level and is not due to problems intrinsic to somatic embryo ontogeny. Studies of ABA concentration in grape embryogenic cultures showed a rapid increase during embryo development, which reach a peak at maturation (Rajasekaran *et al.*, 1982). Cold stratification of somatic embryos results in a rapid decrease in ABA. Exogenously supplied ABA inhibits somatic embryo germination (Gray, 1989). Because ABA is a controlling factor of dormancy in many types of seeds (Bewley and Black, 1985), it could also function in grape somatic embryos. In contrast to ABA, exogenously supplied GA₃ induces grape somatic embryo germination and the concentration of

endogenous GA₃-like compounds increases during cold stratification (Takeno *et al.*, 1983; Pearce *et al.*, 1987). These studies suggest a simple endogenous control of embryo development and germination, whereby ABA inhibits precocious germination and thus promotes normal development while GA₃ promotes germination.

Dehydration can also be used to manipulate grape somatic embryo dormancy (Gray, 1987b, 1990). During dehydration, grape somatic embryos undergo morphological changes similar to those of orchard grass (Gray, 1987b). Their water content equilibrates to 13% when stored at 70% RH, and they resume a normal appearance after rehydration. Genotypic differences in response have been noted; culture lines that produce relatively well-developed somatic embryos are most responsive (Gray, 1989). After 21 days of dehydrated storage, 34% of embryos from one grape genotype produce plants following imbibition (Table 22.1.2). Dehydrated embryos germinate immediately after imbibition, whereas no plants have been recovered from non-dehydrated controls.

Dehydration alters the germination pattern of grape somatic embryos (Gray, 1989). Only somatic embryos that have been dehydrated are capable of germinating and growing in a synchronous pattern of root to shoot emergence. This is in contrast to abnormal germination of non-dehydrated control embryos with and without treatment with dormancy-breaking growth regulators. Although plants are occasionally obtained from the latter type of embryo, notably with BA pre-treatment, root and shoot emergence is not synchronous.

Suspension culture-derived somatic embryos exhibit major differences in morphology, germination characteristics and rate of early plant development (Jayasankar *et al.*, 1999a, 2001a, 2002). Somatic embryos from suspension cultures have large suspensors and reduced cotyledons compared to somatic embryos from semi-solid medium. They also appear to develop precociously, bypassing dormancy, and are capable of rapid plant development. Suspension culture-derived somatic embryos can be placed directly in sand or potting mix for plant

Table 22.1.2. Chronology of selected literature concerning genetic transformation with plant regeneration in grape.

Date	Species/variety	Explant ^a	Vector/strain	Marker gene(s) ^b	Functional gene/trait ^c	Reference
1992	<i>V. rupestris</i> St George	Anther/SE	Agro/CGN7320	NPTII/GUS	—	Mullins et al.
1994	<i>V. rupestris</i>	Petiole/SE	Agro/LBA4404	NPTII/GUS	—	Martinelli and Mandolino
1994	<i>V. vinifera</i> 'Koshusanjaku'	Leaf	<i>A. rhizogenes</i>	NPTII/GUS	—	Nakano et al.
1995	<i>V. rupestris</i> , <i>Vitis</i> hybrid '110 Richter'	Anther/EC	Agro/LBA 4404	NPTII/GUS	GFLV/viral resistance	Krastanova et al.
1995	<i>V. vinifera</i> 'Chardonnay', <i>Vitis</i> hybrids '41B' & 'SO4'	Anther/EC	Agro/LBA 4404	NPTII/GUS	GFLV/viral resistance	Mauro et al.
1995	<i>V. vinifera</i> open pollinated hybrid	Zygotic embryo/SE	Agro/EHA101 & 105	NPTII/GUS	—	Scorza et al.
1996	<i>Vitis</i> hybrid 'Chancellor'	EC susp	Biolistic	NPTII/GUS	—	Kikkert et al.
1996	<i>V. vinifera</i> 'Red Globe'	Anther/EC	Agro/LBA4404	hygromycin	—	Perl et al. (a)
1996	<i>V. vinifera</i> 'Superior Seedless'	Anther/SE	Agro/LBA4404 & GVE3101	BAR or hygromycin	—	Perl et al. (b)
1996	<i>V. vinifera</i> 'Thompson Seedless'	Leaf/SE	Agro/EHA101 & 105	NPTII/GUS	Shiva-1, Tom RSV/microbial and viral resistance	Scorza et al.
1996	<i>Vitis</i> hybrids 'Freedom', 'Teleki 5C', '101-14'	Leaf/SE	Agro/LBA4404	NPTII/GUS	GNA/insect resistance	Viss and Driver
1998	<i>V. vinifera</i> 'Sultana' (= 'Thompson Seedless')	Anther/EC, SE	Agro/EHA101 EHA105	NPTII/hygromycin/GUS	—	Franks et al.
1999	<i>V. vinifera</i> 'Thompson Seedless'	Leaf/SE	Agro/EHA105	NPTII/GFP	—	Li et al.
2000	<i>V. vinifera</i> 'Chardonnay', 'Merlot'	EC susp	Biolistic	NPTII	Chitinase/fungal resistance	Kikkert et al.
2000	<i>Vitis</i> rootstocks & hybrids '3309 C', 'MGT 101-14', 'Riparia Gloire', <i>V. rupestris</i> 'St George', 'Teleki 5C'	Anther/EC	Agro/C58Z707, LBA4404	NPTII/GUS	GFLV, GLRaV-2, GLRaV-3	Krastanova et al.
2000	<i>V. vinifera</i> 'Cabernet Sauvignon', 'Podarok Magarach', 'Rubinovi Magarach', <i>Vitis</i> rootstock 'Krona 42'	Leaf, petiole, stem/primary explant, OR	Agro/?	NPTII/BAR	BAR/herbicide resistance	Levenko and Rubtsova
2000	<i>V. vinifera</i> 'Superior Seedless', <i>V. rupestris</i>	Anther, petiole/SE	Agro/LBA4404	NPTII	GVA, GVB/viral resistance	Martinelli et al.
2000	<i>V. vinifera</i> 'Neo Muscat'	Ov/SE	Agro/LBA4404	NPTII/GUS	Chitinase/fungal resistance	Yamamoto et al.

2001	<i>V. vinifera</i> 'Cabernet Sauvignon', 'Chardonnay', 'Chenin Blanc', 'Muscat Gordo Blanco', 'Riesling', 'Sauvignon Blanc', 'Shiraz'	Anther/EC	Agro/EHA101 EHA105	NPTII/GFP/GUS	—	Iocco <i>et al.</i>
2001	<i>V. vinifera</i> 'Thompson Seedless'	Leaf/SE	Agro/EHA105	NPTII/GFP	Shiva-1/microbial resistance	Li <i>et al.</i> (a,b)

^aEC = embryogenic callus; G–L = leaf from greenhouse-grown plant; I–L = leaf from *in vitro* shoot culture; OR = organogenic culture; P–C = pericarp-derived callus; SE = somatic embryo.
^bBAR = phosphinothricin resistance gene; GFP = green fluorescent protein gene; GUS = glucuronidase gene; NPTII = neomycin phosphotransferase gene.
^cBAR = phosphinothricin resistance; GFLV = grape fan leaf virus; GLRaV-2 = grapevine leafroll associated closterovirus 2; GLRaV-3 = grapevine leafroll associated closterovirus 3; GNA = homopteran insect resistance; GVA = grapevine virus A; GVB = grapevine virus B; Shiva-1 = synthetic lytic peptide; Tom RSV = tomato ringspot virus.

development (Jayasankar *et al.*, 2001b). Somatic embryos that develop on semi-solid medium differ from those from liquid medium with respect to endogenous GA₃ level (S. Jayasankar and Gray, unpublished data); although ABA levels are the same in both types of somatic embryos, the GA₃ level is greater in those from liquid medium. The suspensor is a site of GA₃ biosynthesis and liquid medium-derived somatic embryos have larger suspenders. Conditions that favour larger suspenders could also reduce dormancy by causing higher GA₃ levels, which, in turn, counteract the action of ABA.

5.1.2. Organogenesis

Shoot organogenesis provides an alternative pathway for regenerating plants from cells. In grape, both direct and indirect shoot organogenesis has been reported. Direct organogenesis occurs via adventitious budding from the explant (Litz and Gray, 1992), presumably without intermediate callus formation. Therefore, the shoots may arise directly from predetermined cells. Conversely, indirect shoot organogenesis occurs from callus derived from the primary explant (Litz and Gray, 1992). Indirect organogenesis would be preferred to direct shoot organogenesis when proliferation and maintenance of a specific cell line are required; however, if prolonged culture increases genetic abnormalities, direct regeneration would provide the best possibility for obtaining true-to-type plants. Shoot organogenesis also provides a convenient method for *Agrobacterium*-mediated genetic transformation, since the regenerants would not undergo rejuvenation.

Although adventitious shoots have been induced from somatic embryo explants (Vilaplana and Mullins, 1989), plant regeneration via direct organogenesis has usually been described from young leaves. Barlass and Skene (1978, 1980a,b, 1981) utilized fragmented shoot apices, which were first cultured in drops of liquid MS medium containing BA. Leaf-like structures that developed were transferred on to semi-solid medium after 10 days and adventitious buds formed on swollen basal tissue. These buds elongated into shoots that could be rooted.

This method appears to have broad application since it has been applied to five *V. vinifera* cultivars, *V. champini*, *V. rupestris*, hybrids containing these species and, variously, *V. berlandieri*, *V. labrusca*, *V. longii* and *V. riparia* (Barlass and Skene, 1980b).

Adventitious shoots have been obtained from leaf lamina and petiole explants of *V. vinifera* × *V. labruscana* 'Catawba', with a higher percentage of petioles responding than lamina (Cheng and Reisch, 1989). Stamp *et al.* (1990a,b) utilized apical leaves < 15 mm long from micropropagated plantlets. The leaves were placed on medium with BA, and adventitious shoots were obtained from five *V. vinifera* cultivars, *V. rupestris* and a hybrid between the two species. Shoots originated primarily from the cut petiole surface and from wounded lamina tissue. Histological analysis of regeneration from the cut petiole surface showed that both epidermal and subepidermal cells contributed to shoot meristem development and confirmed that regeneration did not involve a callus stage (Colby *et al.*, 1991). Clog *et al.* (1990) described a similar procedure for several *Vitis* rootstocks, but reported that a short intermediate callus stage occurs from which adventitious buds originate. Martinelli *et al.* (1996) regenerated 18 species and cultivars from leaf tissue.

Shoot organogenesis from callus was obtained by Rajasekaran and Mullins (1981), who cultured internode segments in liquid medium containing 2,4-D, NOA and BA. Under these conditions callus formed that gave rise to adventitious buds. Only tissue from seedlings was responsive. Only seedlings of *V. rotundifolia* and three hybrids between *V. rupestris* and *V. vinifera* produced buds. Rooted shoots were obtained only from the hybrids. Tang and Mullins (1990) described adventitious bud formation from petiole and lamina callus of several *Vitis* species and hybrids. BA together with NAA was more effective than BA with 2,4-D and material obtained from growth chambers and *in vitro* cultures was equally responsive. Adventitious buds of some cultivars could not be induced to root and response rate for many was rather low. Therefore, this regeneration pathway is

currently of limited use when compared to direct organogenesis.

5.1.3. Haploids

Although anther culture has been used to produce apparently diploid embryogenic cultures, there are a few reports of haploid cell proliferation (Gresshoff and Doy, 1974; Sefc *et al.*, 1997) and only one unrepeated report concerning recovery of haploid plants (Zou and Li, 1981) for grape. Sefc *et al.* (1997) reported embryogenesis from several '*Vitis*' subspecies numbered genotypes, but not plant regeneration. However, the depicted 'embryoids' were not convincing as somatic embryos. The lack of progress in this area, despite numerous examples of regeneration of heterozygous diploids from anther culture, suggests that haploidy may be a lethal condition in *V. vinifera* (Mullins, 1990). The potential of fertile dihaploids would be significant, however, since all recessive genes would be expressed and the genetic basis of important traits, e.g. seedlessness, could be more readily determined. This would lead to the development of homozygous breeding lines, allowing desirable traits to be more predictably transmitted through hybridization. Genetic mapping would be greatly simplified using homozygous material. One potential obstacle to the development of dihaploids relates to the severe inbreeding depression that is common in progeny of grape obtained through selfing. Use of dihaploids would be limited if a similar or greater (due to increased homozygosity) reduction in vigour occurred.

5.1.4. Protoplast isolation and culture

Somatic hybridization of grape is dependent on the ability to regenerate plants from protoplasts. There have been two reports of plant regeneration from protoplasts from embryogenic cultures. Reustle *et al.* (1995) described regeneration from protoplasts of *Vitis* hybrid 'Seyval Blanc'. Reustle *et al.* (1995) embedded protoplasts in alginate gell, from which embryogenic cells and somatic embryos emerged. Zhu *et al.* (1997) obtained plants from protoplasts of *V.*

vinifera 'Koshusanjaku' utilizing embryogenic cultures. Their procedure involved embedding of protoplasts in gellan gum droplets. Increased regeneration was observed when the droplets were bathed in liquid medium with activated charcoal.

Plant populations from embryogenic 'Seyval Blanc' protoplasts subsequently were found to exhibit genetic variation (Reustle and Matt, 2000); some individuals were polyploid, i.e. autopolyploid. Furthermore, three of 47 clones evaluated with RAPDs showed altered banding patterns. These observations stress the need for stringent clonal selection and also the opportunities to utilize protoplast culture as a source of genetic variation. The embryogenic protoplasts were also electrofused with non-morphogenic protoplasts and somatic hybrid callus was obtained (Matt *et al.*, 2000), although plant recovery was not reported. Utilization of protoplast technology for genetic improvement of grape, e.g. production of allopolyploids or dihaploids, does not appear to have occurred. Plant regeneration from protoplasts of additional cultivars would permit these routes for genetic improvement to be evaluated.

5.2. Genetic manipulation

5.2.1. Mutation induction and somaclonal variation

Although there has not been a definitive study of the effects of *in vitro* culture on somaclonal variation in grape, several reports have noted obvious differences between original explant donor vines and regenerated plants. Alleweldt (1987) stated that somaclonal variation had not yet been confirmed, but predicted that it would be observed. Differences in leaf morphology and prolonged juvenility have been noted in plants obtained from axillary bud culture (Nozeran *et al.*, 1983; Grenan, 1984). Abnormal growth patterns could be maintained by pruning or reversed through changes in environmental conditions, suggesting that the observed variation was due

to epigenetic factors. Isozyme analysis comparing micropropagated plants with source vines showed no difference in banding pattern, although the amount of enzyme varied (Botta *et al.*, 1990).

Krul and Mowbray (1984) compared somatic embryo-derived vines of *V. vinifera* 'Seyval Blanc' with normally propagated vines and found that those from somatic embryos had darker leaves, reddish vs. green canes and cylindrical rather than conical fruit clusters. Furthermore, cuttings from regenerated vines rooted and grew more rapidly. The vines yielded 5 tons (English) of fruit per acre 3 years after planting. Although the somatic embryo-derived vines differed from existing plantings, they corresponded closely to the original description of 'Seyval Blanc'. The authors suggested that the observed differences were due to elimination of latent viruses during *in vitro* culture. Similarly, somatic embryo-derived plants of 'Sultana' (= 'Thompson Seedless', 'Sultanina') all had red-veined, lobed leaves, a characteristic unlike that of the original plants from which donor tissue was obtained (Franks *et al.*, 1998). This suggested reversion to a juvenile (seedling) phenotype, which may not be unexpected of somatic embryo-derived plants.

Objectives. Selection at the cellular level (*in vitro* selection) or screening for somaclonal variants at the whole plant level can be used to alleviate environmental stress, including disease susceptibility (Daub, 1986; Hammerschlag, 1992). *In vitro* selection is a promising means for producing specific improvements in crops with long life cycles and inbreeding depression. There are several possible specific objectives of *in vitro* selection, especially for the serious diseases of grape. *In vitro* selection could also be used to develop varieties resistant to heavy metals, lime or salt. Lime tolerance is of particular importance in Europe where rootstocks contain parentage from *V. berlandieri*, which is tolerant of calcareous (alkaline) soils (Galet and Morton, 1990). Selection for tolerance of alkaline soil conditions in desirable scion varieties would partially reduce dependence on rootstocks. For *in vitro* selection to be pos-

sible, a selection or screening agent must be available. For example, resistance to a disease with symptoms caused by a toxin may be selectable, since morphogenic cells could be challenged directly by the toxin. In this approach, large cell populations could be efficiently screened for cells that are insensitive to the toxin.

Protocols. Most reports concerning *in vitro* selection have involved cultures on semi-solid medium (Hammerschlag, 1992), in which the selection agent was added to the medium. Semi-solid medium systems can permit the occurrence of 'escapes', i.e. cell masses enlarge and grow away from the selection agent. Embryogenic cultures of grape are ideal for *in vitro* selection since regeneration occurs from single cells so that modified traits can be expressed throughout all tissues of regenerated plants (Gray and Mortensen, 1987; Gray, 1989, 1995). Jayasankar *et al.* (1999a) described a culture system that employed liquid medium and supported high-frequency embryogenesis. Since the cells would be in continuous contact with the selection agent, there would be no opportunity for escapes to occur.

In the past, only diseases with symptoms caused by a toxin were thought to have a strong likelihood of being selectable, since morphogenic cells could be challenged directly by the toxin; however, recent studies indicate that many pathogen-derived components, i.e. proteins or even the crude culture filtrate produced by the pathogen, can elicit broad-spectrum defence in plants (Strobel *et al.*, 1996; Jayasankar and Litz, 1998). In this approach, large cell populations could be efficiently screened for cells that are insensitive to the selection agent (Jayasankar *et al.*, 1999b). Since grape is vegetatively propagated, any type of stable resistance obtained in regenerated plants would be of use, regardless of whether or not it was heritable. The selection agent is added to rapidly growing cultures at a predetermined lethal or sublethal dose. Cultures are maintained during one or more cycles of selection. Cells that are resistant to the selection agent proliferate and putatively resistant plants are recovered (Jayasankar *et al.*, 2000).

Accomplishments. Mauro *et al.* (1988) demonstrated that extracts of *E. lata* (Pers.: Fr.) Tul. caused necrosis of plants *in vitro*, but morphogenic cell cultures were not utilized so that selection was not possible. Selection for NaCl tolerance was studied using embryogenic cultures of *V. rupestris* (Lebrun *et al.*, 1985) and shoot cultures of *V. champini* 'Ramsey' and *V. vinifera* 'Cabernet Sauvignon', but selected tolerant cell and tissue cultures did not yield salt-tolerant plants. Long-lived culture lines were not used in these studies, so that it was not possible to subject the cultures to long-term selection pressure.

Jayasankar *et al.* (1998, 2000) demonstrated that *in vitro* selection could be used to regenerate anthracnose-resistant somaclonal variants. Embryogenic 'Chardonnay' suspension cultures were recurrently selected with crude culture filtrate produced by the fungus *E. ampelina*. Selected resistance is broad-spectrum since these cultures also inhibit growth of *Fusarium* spp. in dual culture assays. Molecular analysis revealed that such induced resistance is mediated by constitutive expression of several pathogenesis-related (PR) proteins, e.g. chitinase and thaumatin-like proteins (Jayasankar *et al.*, 1999b, 2000). One of the latter proteins, *Vitis vinifera* thaumatin-like protein (VVTL-1), is antifungal (Jayasankar *et al.*, 2003b). VVTL-1 is highly expressed in embryogenic cultures of several cultivars after *in vitro* selection against culture filtrate produced by two different pathogens, *E. ampelina* and *Colletotrichum gloeosporioides*. Therefore, it may have a much broader role in disease resistance of grape (S. Jayasankar and D.J. Gray, unpublished data). Apart from such PR proteins, grape cell cultures also have detoxifying mechanisms, and genes encoding such detoxifiers have been characterized. For instance, *E. lata*, a fungus causing black arm disease, has been shown to produce a toxin, eutypine (Deswarte *et al.*, 1996). Colrat *et al.* (1999) identified an enzyme, nicotinamide adenine dinucleotide phosphate (NADPH)-dependent aldehyde reductase, in grape, which is a detoxifying agent of eutypine. This enzyme is also present in other species, e.g. *Vigna radiata*, and the gene that

encodes this enzyme is capable of conferring resistance to eutypine (Guillen *et al.*, 1998). Use of purified toxins such as eutypine could complement *in vitro* selection against other pathogens. *In vitro* selection is a useful tool for gene discovery.

Pierce's disease symptoms include marginal leaf burn and limited wilting in mild cases to widespread wilting, rapid dieback and death in extreme instances. Since marginal burn is often associated with a toxin, a number of early studies were aimed at establishing the role of toxin in the disease process. Phytotoxic activity caused by the Pierce's disease bacterium (Lee *et al.*, 1982) has been attributed to culture medium components (Goodwin *et al.*, 1988). High-molecular-weight, cell-free fractions from spent desalted culture medium have also been shown to cause marginal burn in leaf assays (D.L. Hopkins, personal communication). Control treatments consisting of desalted, fractionated fresh medium do not produce marginal burn. Uncertainty about the involvement of a phytotoxin in Pierce's disease has deterred any further research. *Vitis* appears to have Pierce's disease resistance genes (Mortensen, 1968; Mortensen *et al.*, 1977), which indicates that *in vitro* selection using *Xylella* culture filtrate could be achieved. Successful application of this approach would allow the geographic range of pure *V. vinifera* varieties to be expanded into areas of endemic Pierce's disease occurrence and provide a method for disease management in newly affected grape-growing regions.

5.2.2. Somatic hybridization

Somatic hybridization could be utilized to circumvent the sterility inherent in crosses between *Euvinis* and *Muscadinia*. Somatic *Euvinis* × *Muscadinia* hybrids would possess a full, even-numbered complement of chromosomes. Since colchicine-induced polyploidy of *Euvinis* × *Muscadinia* hybrids results in fertile allopolyploids (Jelenkovic and Olmo, 1969), protoplast fusion should overcome sexual incompatibility and sterility based on unequal chromosome numbers. Parthenocarpic seedlessness, which occurs in several *V. vinifera* cultivars and is not fully

accessible with conventional breeding, could be utilized for grape germplasm improvement by somatic hybridization.

5.2.3. Genetic transformation

Objectives. In the major wine-producing areas, classical wine grape cultivars are an integral part of the economy and cannot be easily displaced by new cultivars. Directed genetic modification of existing cultivars by the introduction of single genes would result in improved genotypes that could be accepted as variants of the original cultivars. Thus, genetic transformation offers significant opportunities for the improvement of grape while allowing the continued use of traditional cultivars of considerable economic significance. Possible applications of genetic transformation include virus resistance by integration of viral coat protein genes (Stark and Beachy, 1989; Nejdat *et al.*, 1990), resistance to insect pests by incorporation of genes for *Bacillus thuringiensis* toxin (Barton *et al.*, 1987; Delannay *et al.*, 1989) and herbicide tolerance (Shah *et al.*, 1986; Oxtoby and Hughes, 1990). Other possible applications of transgenic technology include modification of quality and physiological traits, such as seedlessness, antioxidant levels and browning.

Protocol. Significant progress in grapevine transformation has been made (Perl and Eshdat, 1998; Martinelli and Mandolino, 2001; Gray *et al.*, 2002). Genetic transformation has become relatively routine for 'Chardonnay', 'Merlot', 'Superior Seedless' and 'Thompson Seedless' (= 'Sultana', 'Sultanina'). Transformation has been facilitated by refinement of genetic vectors that efficiently express the visual reporter genes, glucuronidase (GUS), green fluorescent protein (GFP) (Table 22.1.2) and luciferase (Hanson *et al.*, 1999) as well as the selectable marker genes BAR, hygromycin and NPTII.

The two commonly used methods of transformation, biolistic-mediated and *Agrobacterium*-mediated, have both been applied to grape. Biolistic bombardment resulted in intense transient GUS expression in somatic embryos of *V. vinifera* 'Thompson Seedless'

(Gray *et al.*, 1993) and has been used to produce transgenic plants from embryogenic suspension cultures of 'Chardonnay', 'Merlot' and 'Chancellor' (Kikkert *et al.*, 1996, 2000). *Agrobacterium*-mediated transformation has been utilized more frequently, including several cultivars of *V. vinifera* (Nakano *et al.*, 1994; Krastanova *et al.*, 1995; Mauro *et al.*, 1995; Scorza *et al.*, 1995, 1996; Perl *et al.*, 1996a,b; Torregrosa and Bouquet, 1997; Franks *et al.*, 1998; Li *et al.*, 1999, 2001a,b,c; Yamamoto *et al.*, 2000; Iocco *et al.*, 2001).

Agrobacterium-mediated transformation of organogenic cultures has been reported for the rootstock *V. rupestris* (Mullins *et al.*, 1990) and for three *V. vinifera* scions and a rootstock (Levenko and Rubstova, 2000). Co-transformation with *A. tumefaciens* and *A. rhizogenes* has been used to target *in vitro* whole plants, since *A. rhizogenes* produces hairy roots from such tissues; however, transgenic plants were not obtained (Torregrosa and Bouquet, 1997). Nakano *et al.* (1994) transformed *V. vinifera* 'Koshusanjaku' with *A. rhizogenes*.

Somatic embryos of grapevine proliferate via direct secondary embryogenesis from single epidermal or subepidermal cells (Gray, 1992, 1995; Gray *et al.*, 2002). Therefore, somatic embryos are ideal targets for transformation, since the regenerative cells are accessible to *Agrobacterium* and single cell origin should result in non-chimeric transformants. Most reports of grapevine transformation have utilized somatic embryos as explants (Table 21.1.2), although embryogenic cultures have also been used for several cultivars (Franks *et al.*, 1998; Iocco *et al.*, 2001).

Tissue necrosis as a result of *Agrobacterium* infection of *V. vinifera* causes inhibition of cell growth, leading to cell death. Transformation efficiencies, the number of transgenic lines and plants recovered have been relatively low compared to other *Vitis* spp., e.g. *V. rupestris* (Krastanova *et al.*, 1995; Mauro *et al.*, 1995; Xue *et al.*, 1999). Perl *et al.* (1996b) described tissue browning and necrosis as a hypersensitive response to *Agrobacterium* infection and demonstrated that onset of browning was correlated with elevated levels of peroxidase activity. Use of

antioxidants such as dithiothreitol (DTT) or polyvinylpolypyrrolidone (PVPP) reduces tissue browning; however, these antioxidants contribute to the inability of kanamycin at concentrations of up to 500 mg/l to inhibit the cell growth of non-transformed grapevine cells. Scorza *et al.* (1996) incorporated high levels of cysteine into culture medium. Incorporation of an explant preconditioning treatment prior to transformation inhibits browning and facilitates recovery of transgenic plants.

Accomplishments. Much transgenic research on grapevine concerns optimization of protocols for a range of genotypes (Iocco *et al.*, 2001). A fusion reporter marker containing the enhanced green fluorescent protein (EGFP) and NPTII genes appears to facilitate transformation of grapevine by allowing both genes to be expressed by a single promoter (Li *et al.*, 1999, 2001a,b). This allowed variation in gene expression due to the use of several promoters and terminators to be eliminated (Li *et al.*, 2001c). The fusion marker also allowed for convenient analyses of tissue-specific expression due to independent transformation events (Gray *et al.*, 2003). Therefore, plants with high transgene expression in xylem tissue could be selected to facilitate research in the development of plants that are resistant to Pierce's disease using the functional genes described below. The fusion marker was used to investigate and optimize factors for *Agrobacterium*-mediated transformation of several grapevine species and varieties.

Recovery of transgenic plants has been categorized in Table 22.1.2. Most emphasis is on resistance to virus diseases using virus-derived genetic elements, e.g. coat protein genes. Xue *et al.* (1999) inserted virus-derived genes into five rootstocks. Transgenic grapevines are being evaluated for resistance to grapevine fanleaf virus (GFLV), a nepovirus found in most grape-growing regions (Barbier *et al.*, 2000), as well as grapevine chrome mosaic nepo virus (Le Gall *et al.*, 1994). Transgenic plants have been recovered with coat protein genes from grapevine viruses A and B (GVA and GVB), which are believed to be involved in Kober

stem grooving and corky bark (Golles *et al.*, 2000; Martinelli *et al.*, 2000). Work is ongoing to produce plants with resistance to arabis mosaic virus (Golles *et al.*, 2000; Spielmann *et al.*, 2000) and grapevine leafroll virus (Krastanova *et al.*, 2000).

Resistance to fungal and bacterial diseases, as well as herbicide resistance, is being addressed. Insertion of a gene encoding a lytic peptide with antimicrobial properties into 'Thompson Seedless' has been reported (Scorza *et al.*, 1996). Lytic peptides were detected in transgenic plants via enzyme-linked immunosorbent assay (ELISA) (Li *et al.*, 2001d). Grapevines containing chitinase and endochitinase genes are being tested for resistance to grey mould and powdery mildew (Harst *et al.*, 2000; Kikkert *et al.*, 2000). Yamamoto *et al.* (2000) have recovered plants that express chitinase with resistance to powdery mildew.

Levenko and Rubtsova (2000) reported using the *Bar* gene to develop resistance of grapevines to the herbicide phosphinothricin (Basta). Transgenic plants were resistant to 20 ml/l Basta. Gollop *et al.* (2000) studied proanthocyanidin biosynthesis by transforming plants to contain genes for dihydroflavonol reductase (DFR) and leucoanthocyanidin dioxygenase (LDOX). Thomas *et al.* (2000) are working to silence polyphenol oxidase (PPO) genes in plants to control browning for raisins. Plants in the field are waiting to be evaluated for PPO activity and dried fruit colour. Grapevines with anti-freezing genes are being evaluated to expand the adaptability of grape to colder regions (Tsvetkov *et al.*, 2000).

6. Germplasm Conservation

Genetic diversity among clonally propagated crops is diminishing due to several factors, e.g. monoculture and urbanization (Gray and Compton, 1993). Vegetatively propagated plants are maintained as field gene banks, which are costly and under constant threat by pests, diseases and adverse climatic conditions (Towill, 1988; Withers, 1989).

Shoot tip and nodal cultures of grapevine are suitable for germplasm conservation, if they can be kept under slow-growth conditions and can survive for long periods of time without transfer. Alleweldt and Harst-Langenbucher (1987) showed that cultures treated with Cycocel can survive for up to 10 months at 3°C without loss of viability. Skene *et al.* (1988) demonstrated that shoot tip and nodal cultures can survive for up to 12 months at 9.5°C. Moriguchi and Yamaki (1989) compared cultures kept at 5 or 10°C with those grown at 28°C, but on medium with low ammonium nitrate levels, for up to 290 days. They reported a higher survival rate with the latter treatment. D.J. Gray (unpublished data) has routinely maintained shoot tip and nodal cultures of *V. vinifera* cultivars, *Vitis* spp. and interspecific hybrids on MS medium with 2.5 µM BA and 3% sucrose at 6°C for > 27 months. The cultures were in an active growth phase prior to cold storage.

Use of synthetic seed for germplasm conservation of grape has potential for storage of clonal germplasm in seed repositories (Gray, 1989, 1990; Gray and Purohit, 1991a,b). More genotypes could be conserved since space problems would be eliminated. Grape somatic embryos can be dehydrated and stored at 4°C for at least 3 years with no loss of viability (S. Jayasankar and D.J. Gray, unpublished data).

Shoot tip and nodal cultures of several *Vitis* hybrids and *V. vinifera* cultivars have been cryopreserved (Plessis *et al.*, 1991, 1993; Wang *et al.*, 2000). Tissue was encapsulated and dehydrated before cooling. A two-step cooling protocol was better than vitrification (Plessis *et al.*, 1993) and thawing was found to significantly influence survival (Wang *et al.*, 2000). Up to 60% survival of cryopreserved material was obtained.

Grapevine somatic embryogenesis has been recognized as a potential tool for germplasm conservation (Mullins and Srinivasan, 1976; Gray, 1987d, 1989, 1990, 1995; Gray and Purohit, 1991a,b; Gray and Compton, 1993). In grapevine, ultralow-temperature cryopreservation of suspension-

grown embryogenic cultures from different cultivars has been demonstrated with varying degrees of success (Dussert *et al.*, 1991, 1992; Wang *et al.*, 2002). Dussert *et al.* (1991) used a two-step gradual cooling procedure to preserve embryogenic cell cultures. Wang *et al.* (2002) utilized encapsulation and dehydration of embryogenic cell cultures with a single-step cooling procedure. Despite these successes with cryopreservation of embryogenic cells, well-developed somatic embryos that exhibit dormancy, i.e. synthetic seed, may be better suited for germplasm storage, since they function like the zygotic embryos commonly stored in seed repositories (Gray, 1995).

6. Conclusions

Embryo rescue technology is already in widespread use for seedless grape development. Identification and use of molecular markers have already been a significant aid for germplasm maintenance and have allowed the origin of certain cultivars to be determined with precision and certainty. Improved methods of germplasm conservation and vegetative propagation are possible using synthetic seed technology. *In vitro* selection for desirable traits in established cultivars shows immediate promise. Although haploids and dihaploids have not been obtained, regeneration from embryogenic protoplasts is significant for cell manipulation. Genetic transformation appears to be relatively commonplace for a number of important cultivars, and potentially useful genes are available to test in grape. It remains to be seen if transgenic modifications of existing cultivars will retain their unique phenotypes. The promise of biotechnology is finally ready to be definitively tested for grape cultivar improvement.

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Index

- ACC (1-aminocyclopropane-1-carboxylic acid)
 - enzymes, 45, 160, 180, 194–195, 482, 519
- Actinidia* spp. *see* kiwifruit
- Actinidiaceae*, 1
 - see also* kiwifruit
- actinidin, 4, 8
- acyl carrier proteins, 121
- adverse effects, 4, 29, 32
 - see also* allergenicity
- agriculture
 - production needs, xx
- allergenicity, 479, 483, 520
 - see also* adverse effects
- almond
 - allergenicity, 520
 - botany and history, 513
 - cryopreservation, 531
 - cultivar identification, 521
 - economic and nutritional importance, 514
 - genetic transformation, 529(tab), 530
 - linkage mapping, 521
 - organogenesis, 526
 - rootstocks, 516
 - scions, 517, 518
 - seed proteins, 520
 - self-incompatibility, 522
 - virus elimination, 523
- alternate oxidase, 46
- aluminium, resistance to, 190(tab), 195
- American Chestnut Co-operators Foundation, 270
- American Chestnut Foundation, The, 270
- Anacardiaceae*, 29
 - see also* cashew; mango
- Anacardium occidentale* *see* cashew
- Ananas comosus* *see* pineapple
- Annona* spp.
 - botany and history, 74
 - control of ripening, 76
 - diseases, 75, 76
 - DNA markers, 77–78
 - economic importance, 74–75
 - fruit quality, 76–77
 - gene cloning, 77
 - genetic transformation, 81
 - haploid recovery, 80
 - isozyme markers, 77
 - micropropagation, 78–79, 82–84(tab)
 - organogenesis, 79–80, 82–84(tab)
 - pollination, 75
 - protoplast isolation and culture, 81, 83(tab)
 - ripening, 76, 81
 - rootstocks, 75
 - scions, 75–77
 - tree architecture, 76
 - triploid recovery, 80–81
- Annonaceae*, 73
 - see also* *Annona* spp.
- anthocyanins, 482
- anthracnose, 32, 53, 629, 693
- anticancer agents *see* cancer: prevention and inhibition
- antioxidants, 223, 481, 514
- apical shoot necrosis, 51–52
- apomixis, facultative, 570, 586
- apple
 - allergenicity, 483
 - anthocyanins, 482
 - antioxidants, 481
 - botany and history, 475–476
 - breeding and genetics: reviews, 476–477
 - cold tolerance, 480
 - cryopreservation, 496
 - Durable Applied Resistance in Europe programme, 481
 - economic importance, 476
 - flowering, 483–484
 - fruit quality, 479, 481, 487
 - functional genomics, 488
 - gene cloning, 482–484
 - genetic transformation, 492–496
 - germplasm, 476
 - gibberellins, 483
 - haploid recovery, 490–491

apple *continued*

- incompatibility (self and cross), 482, 485, 492
- industrial partnerships, 496–497
- low-allergenic cultivars, 483
- marker-assisted selection, 484–488
- micropropagation, 488–489
- mutagenesis and somaclonal variation, 491–492
- organogenesis, 490
- pests and diseases, 477, 479–480, 487–488, 492, 493–494
- polyphenol oxidase, 483
- protoplast isolation and culture, 491
- QTL analysis, 481
- ripening, 482
- rootstocks, 477–478
- scions, 479–482
- skin colour, 492
- somatic embryogenesis, 489–490
- sorbitol synthesis, 483
- storage disorders, 481
- superficial scald, 483
- tree architecture, 480
- virus elimination, 489

apricot

- allergenicity, 520
- botany and history, 513
- cultivar identification, 521
- economic importance, 514
- genetic transformation, 529(tab), 530
- micropropagation, 523
- mutagenesis and somaclonal variation, 527
- organogenesis, 526
- rootstocks, 516
- scions, 517, 518
- self-incompatibility, 522

architecture, tree *see* tree architecture*Arecaceae*, 89

see also coconut palm; date palm; oil palm

Artocarpus spp.

- AFLP markers, 353
- botany and history, 351–352
- breeding and genetics, 352
- medical properties, 352
- micropropagation, 355
- organogenesis, 356
- RFLP markers, 353

Asian pear *see* pearatemoya *see* *Annona* spp.

auxins: metabolism during rooting, 333–334

Averrhoa carambola *see* carambola

avocado

- botany and history, 326
- cryopreservation, 340–341
- dwarfing, 327, 328
- economic importance, 327
- fruit quality, 328

- genetic markers, 330–331
- genetic transformation, 339–340
- heterozygosity assessment, 330–331
- micropropagation, 332–335
- mutagenesis and somaclonal variation, 338
- pests and diseases, 328, 329, 338–339, 341
- protoplast isolation and culture, 337–338
- ripening, 328–329, 330, 340
- rootstocks, 327–328
- salinity stress tolerance, 328
- scions, 328–329
- somatic embryogenesis, 335–337
- somatic hybridization, 338–339
- tree architecture, 329
- virus elimination by micrografting, 335

BAC (bacterial artificial chromosome) libraries, 369, 462, 522, 595, 678

banana and plantain

- BAC libraries, 369
- banana streak badnavirus, 371
- botany and history, 366–367
- breeding and genetics, 367–368
- cryopreservation, 382
- dwarfism, 370
- economic and social importance, 367
- gene cloning, 370–371
- genetic transformation, 378–381
- genome, 368–369
- germplasm conservation, 381–382
- micropropagation, 371–374
- mutagenesis and somaclonal variation, 376–378
- pests and diseases, 367, 368, 378
- protoplast isolation and culture, 377
- RAPD markers, 370
- ripening, 379
- somatic embryogenesis, 374–376, 380(fig.)
- somatic hybridization, 378
- virus elimination, 374–375

bayoud disease, 145–146, 151

biodiversity, xxiii

biolistics, 131, 166, 284, 285(fig.), 379–380, 448–449

biotechnology

- consumer perceptions, 558–559
- impact on diet quality and safety, xx

black pod, 642, 647(tab), 648–649

blackberry *see* cane fruit

blackheart, 160–161

blueberry

- botany and history, 222–223
- breeding and genetics, 223–225
- cold tolerance, 225–228, 233–234
- cryopreservation, 239
- dehydrins, 225–228, 233–234
- DNA fingerprinting, 231–232
- economic and nutritional importance, 223

- EST-PCR markers, 230–231, 232–233
fruit quality, 224, 225
functional genomics, 233–234
gene cloning, 225–228
genetic mapping, 232–233
genetic transformation, 238–239
ISSR markers, 230
K segments, 225, 226, 227
marker-assisted selection, 228
micropropagation, 234–236
microsatellites, 230
mutagenesis and somaclonal variation, 237–238
organogenesis, 236–237
pests and diseases, 224
protein markers, 229
RAPD, 230, 231–232, 233
RFLP, 229–230
- bombardment, microprojectile *see* biolistics
- botany and history
 almond, 513
 Annona spp., 74
 apple, 475–476
 apricot, 513
 Artocarpus spp., 351–352
 avocado, 326
 banana and plantain, 366–367
 blueberry, 222–223
 cacao, 639–642
 cane fruit, 566–569
 carambola, 430
 cashew, 30
 cherry, 513
 chestnut, 265–267
 citrus, 584–585
 coconut palm, 90–91
 cranberry, 247
 date palm, 144
 fig, 350–351
 grape, 672–673
 guava, 394
 kiwifruit, 2–3
 litchi and longan, 628
 mango, 40–41
 mangosteen, 211
 mulberry, 350
 oil palm, 113
 olive, European, 404–405
 papaya, 174
 passionfruit, 436–438
 peach and nectarine, 512
 pear, 543
 pecan, 298
 pineapple, 158–159
 pistachio, 62–63
 plum, 512–513
 strawberry, 456
 walnut, 307–308
- bramble *see* cane fruit
- breadfruit *see* *Artocarpus* spp.
- breeding: general objectives, xxii–xxiii
- Bromeliaceae*, 157
 see also pineapple
- cab* promoter (peach), 520
- cacao
 botany and history, 639–642
 breeding and genetics, 642–644
 classification, 640, 641
 cryopreservation, 660
 economic importance, 642
 gene cloning, 644–645
 genetic transformation, 659–660
 haploid recovery, 658
 linkage maps, 645–646, 647(tab)
 marker-assisted selection, 645–650
 micropropagation, 650–651
 mutagenesis and somaclonal variation, 658–659
 pests and diseases, 642–643, 647(tab), 648–649
 protoplast isolation and culture, 658
 somatic embryogenesis, 651–652, 653–655(tab), 656–658
 viruses, 642, 659, 660
 yields, 643, 646, 648
- cancer: prevention and inhibition, 3
- cane fruit
 botany and history, 566–569
 breeding and genetics, 569–570
 cryopreservation, 577
 facultative apomixis, 570
 gene cloning, 570–571
 genetic transformation, 576–577
 genome mapping, 572–573
 markers, 571–572
 micropropagation, 573
 organogenesis, 573–574
 pests and diseases, 576, 577
 protoplast isolation and culture, 574
 ripening, 576
 somaclonal variation, 574–576
 somatic embryogenesis, 573
 triploid recovery, 574
 virus elimination, 573
 virus resistance, 570, 576–577
- carambola
 botany and history, 430
 breeding and genetics, 430–431
 economic importance, 430
 organogenesis, 432
 oxalic acid, 431
 postharvest losses, 431
- cardol, 32
- Carica papaya* *see* papaya

- Caricaceae*, 173
 see also papaya
- Carizzo citrange, 608, 610
- Carya illinoensis* *see* pecan
- cashew
 botany and history, 30
 dwarfism and precocity, 32
 economic importance, 30–31
 haploid recovery, 36
 micrografting, 34
 micropropagation, 33–34
 molecular genetics, 33
 organogenesis, 34–35
 pests and diseases, 31, 32, 33
 quality of fruit and nuts, 32, 33
 rootstocks, 31
 scions, 31–33
 somatic embryogenesis, 35–36
- cashew nut shell liquid, 31, 32
- Castanea* spp. *see* chestnut
- cell culture: general remarks, xxi
- cell walls: synthesis and turnover, 46–47
- cellulase, 329
- chalcone synthase, 315
- cherimoya *see* *Annona* spp.
- cherry
 allergenicity, 520
 botany and history, 513
 cryopreservation, 531
 cultivar identification, 522
 economic and nutritional importance, 514
 genetic transformation, 528–529, 529(tab), 530
 haploid recovery, 526
 linkage mapping, 521
 mutagenesis and somaclonal variation, 527
 protoplast isolation and culture, 526–527
 scions, 517, 518
 somatic embryogenesis, 524–525
 somatic hybridization, 554–555
 virus elimination, 523
- cherry leafroll virus, 310, 313
- chestnut
 botany and history, 265–267
 breeding and genetics, 268–269
 cryopreservation, 285, 286(fig.)
 economic and nutritional importance, 267
 genetic manipulation, 284
 genomics, 272
 in vitro axillary enhancement, 273–275(tab)
 micropropagation, 272–276
 molecular markers, 270–272
 nut quality, 269
 organogenesis, 282–284
 pests and diseases, 268–270
 somatic embryogenesis, 277–281, 282(fig.)
- chestnut blight canker, 268–270
- chitinases, 593
- chloroplasts: DNA
 cane fruit, 571–572
 citrus, 587–588
 European olive, 409
 kiwifruit, 3
 mangosteen, 214
 passionfruit, 440
- chlorosis, iron, 545
- chocolate, 640
- citrange, Carizzo, 608, 610
- citron *see* citrus
- citrus
 acid accumulation, 592
 BAC (bacterial artificial chromosome) libraries, 595
 botany and history, 584–585
 chitinases, 593
 cold tolerance, 592–593
 cryopreservation, 611
 DNA markers, 587–589
 economic importance, 585
 EST (expressed sequence tag) libraries, 596
 flavonoids, 592
 gene cloning, 591–594, 595–596
 genetic transformation, 605–611
 haploid recovery, 599
 isoenzyme polymorphism analysis, 587, 588
 linkage mapping, 594–595
 marker-assisted selection, 594
 micropropagation, 596
 mutagenesis and somaclonal variation, 601–602
 organogenesis, 599
 pests and diseases, 595–596, 605
 protoplast isolation and culture, 601
 QTL analysis, 594–595
 ripening, 592
 rootstocks, 590
 scions, 591
 somatic embryogenesis, 597–599
 somatic hybridization, 603–605
 stress, 592
 taxonomy and genetic diversity, 585–590
 triploid production, 599–601
 virus elimination by micrografting, 596–597
 viruses, 595
- citrus tristeza virus (CTV), 595
- Clematoclethra*, 1
- cloning, gene
 Annona spp., 77
 apple, 482–484
 avocado, 329–330
 banana and plantain, 370–371
 blueberry, 225–228
 cacao, 644–645
 cane fruit, 570–571
 citrus, 591–594, 595–596

- European olive, 406–408
- grape, 677–678
- kiwifruit, 6
- mango, 44–47
- mulberry, 352–353
- oil palm, 120–123
- papaya, 179–181
- pear, 546–547
- pineapple, 160–161
- Prunus* spp., 518–520
- strawberry, 458–459
- walnut, 314–315
- cloudberry *see* cane fruit
- Clusiaceae*, 209
 - see also* mangosteen
- CNSL (cashew nut shell liquid), 31, 32
- coconut oil, 92
- coconut palm
 - botany and history, 90–91
 - breeding, 92–93
 - COGENT (Coconut Genetic Resources Network), 98, 102(tab)
 - conservation, 98–103
 - diseases, 100, 101(tab), 102(tab)
 - economic importance, 91–92
 - embryo storage, 99
 - genetic markers, 94
 - guidelines for safe movement of germplasm, 100
 - haploid recovery, 98
 - International Coconut genebank, 100, 102, 103(tab)
 - marker-assisted selection, 94
 - pollination, 93, 99–100
 - protoplast isolation and culture, 98
 - somatic embryogenesis, 03, 95–98
- Cocos nucifera* *see* coconut palm
- codling moths, resistance to, 317–318
- COGENT (Coconut Genetic Resources Network), 98, 102(tab)
- cold, tolerance to
 - apple, 480
 - blueberry, 225–228, 233–234
 - citrus, 592–593
 - European olive, 416
 - papaya, 177–178, 181, 190(tab)
 - pear and quince, 544, 545, 546
 - strawberry, 468
- conservation
 - coconut palm, 98–103
- consumers
 - familiarity with pear cultivars, 558
 - perceptions of biotechnology, 558–559
- coronary heart disease, 315
- cranberry
 - botany and history, 247
 - breeding, 248, 257–258
 - economic and nutritional importance, 247–248
 - embryo rescue, 252
 - genetic transformation, 252–256
 - herbicide tolerance, 255–256
 - in vitro* storage, 257
 - insect resistance, 253–255
 - micropropagation, 249–250, 251(fig.)
 - molecular genetics, 248–249
 - National Clonal Germplasm Repository (NCGR), USA, 257
 - organogenesis, 250–251
 - pigmentation, 248
 - ploidy manipulation, 251–252
- crops, primary food, xix
- crown gall disease, 310–311, 318
- cryopreservation
 - apple, 496
 - avocado, 340–341
 - banana and plantain, 382
 - blueberry, 239
 - cacao, 660
 - cane fruit, 577
 - chestnut, 284–285, 286(fig.)
 - citrus, 611
 - coconut palm, 99
 - coconut pollen, 100
 - date palm, 152
 - European olive, 418
 - grape, 696
 - kiwifruit, 19
 - litchi and longan, 634
 - mango, 56
 - mulberry, 358
 - oil palm, 134
 - papaya, 195–196
 - pear, 557–558
 - pecan, 304
 - pineapple, 167
 - Prunus* spp., 531
 - strawberry, 468
 - walnut, 318
- Cryphonectria parasitica*, 268–270
- culture, cell and tissue: general remarks, xxi
- currency, seeds as, 640
- Cydonia* spp. *see* quince
- cystatins, 181
- cytochrome P-450, 329–330
- date palm
 - botany and history, 144
 - breeding objectives, 145–146
 - cryopreservation, 152
 - DNA markers, 147
 - haploid recovery, 151
 - micropropagation, 147
 - mutation induction, 151

- date palm *continued*
 pests and diseases, 145–146, 151
 protein markers, 146–147
 regeneration, 147–148
 organogenesis, 150–151
 somatic embryogenesis, 148–150
 socio-economic importance, 144
- defensins, 557
- dehydrins, 225–228, 233–234, 520
- depression, inbreeding, 674
- desaturases, 120–121, 122
- Dimocarpus longan* *see* longan
- diseases *see* pests and diseases
- DNA, chloroplast *see* chloroplasts: DNA
- DNA: hypomethylation, 128–129
- DNA markers
 AFLP
 Annona spp., 77–78
 apple, 485
 Artocarpus spp., 353
 coconut palm, 94
 European olive, 409
 oil palm, 115
 papaya, 182–183
 strawberry, 460
 walnut, 315
 chestnut, 271(tab)
 EST-PCR, 230–234
 ISSR (inter-simple sequence repeat) markers
 apple, 486
 blueberry, 230
 citrus, 588, 589
 European olive, 410
 strawberry, 460
 walnut, 315
 ISTR (inverse sequence-tagged repeat), 94
 M13 bacteriophage probe, 571
 microsatellites
 Annona spp., 78
 avocado, 330–331
 blueberry, 230
 coconut palm, 94
 grape, 677–678
 kiwifruit, 3
 oil palm, 115–117
 walnut, 315
 minisatellites, 571
 pear and quince, 547, 549(tab), 550
Prunus spp., 520–523
 RAPD
 Annona spp., 77
 apple, 484–485
 avocado, 331
 banana and plantain, 370
 blueberry, 230, 231–232, 233
 cacao, 641
 cane fruit, 572
 cashew, 33
 chestnut, 272
 citrus, 587, 588–589, 594
 coconut palm, 94
 cranberry, 248–249
 date palm, 147
 European olive, 410
 fig, 353
 kiwifruit, 6–7
 litchi, 630
 mango, 43–44, 54
 mangosteen, 214
 oil palm, 115
 papaya, 182
 passionfruit, 441
 pineapple, 161
 pistachio, 65
 strawberry, 459–460, 461
 RFLP
 Annona spp., 77
 apple, 484
 Artocarpus spp., 353
 avocado, 331
 blueberry, 229–230
 cacao, 641
 chestnut, 271–272
 citrus, 587
 date palm, 147
 European olive, 409, 410
 kiwifruit, 3
 oil palm, 114, 115
 pineapple, 161–162
 RGA (resistance gene analogues), 486–487
 SCAR
 apple, 485
 citrus, 587, 588, 589
 European olive, 410
 kiwifruit, 6–7
 papaya, 182
 SSR
 apple, 485–486
 grape, 677–678
 transposable elements, 486
see also chloroplasts: DNA; selection, marker-assisted
- dormancy, somatic embryo, 684
- drought, tolerance to, 212
- Durable Applied Resistance in Europe
 programme, 481
- dwarfism
Annona spp., 75
 avocado, 327, 328
 banana and plantain, 370
 cashew, 32
 coconut palm, 93
 guava, 396
 pear, 557
Dysaphis devecta, 480

- Elaeis guineensis* see oil palm
Elaeis oleifera, 116, 121
embryogenesis, somatic
 apple, 489–490
 avocado, 335–337
 banana and plantain, 374–376, 380(fig.)
 cacao, 651–652, 653–655(tab), 656–658
 cane fruit, 573
 cashew, 35–36
 chestnut, 277–281, 281(fig.), 282(fig.)
 citrus, 597–599
 coconut palm, 95–98, 103
 date palm, 148–150
 European olive, 412, 414
 grape, 680–687, 690, 696–697
 selected literature, 682–684(tab)
 guava, 397–398, 399(fig.)
 kiwifruit, 10
 litchi and longan, 630–632
 mango, 47–52
 mangosteen, 214
 mulberry, 356
 oil palm, 123–128
 papaya, 184–185
 passionfruit, 443–444
 pecan, 300–302
 pineapple, 164
 pistachio, 67–68
 Prunus spp., 524–525
 strawberry, 463
 walnut, 316–317
embryony, nucellar, 586
embryos, rescue of
 cranberry, 252
encapsulation, embryonic
 apple, 496
 coconut palm, 99
 grape, 696–697
 guava, 398
 pistachio, 69
environment: impact of biotechnology, xx
Ericaceae, 221
 see also blueberry; cranberry
EST (expressed sequence tag) libraries, 369, 486, 596, 678
EST-PCR markers see under DNA markers
ethylene
 regulation of biosynthesis, 45
European olive see olive, European
European Union, 162

facultative apomixis, 570, 586
Fagaceae, 263
 see also chestnut
fatty acid synthetase, 130(fig)
fatty acids, 130–131
 biosynthesis, 130–131, 315, 406

Ficus carica see fig
fig
 botany and history, 350–351
 breeding and genetics, 352
 economic importance, 351
 isoenzyme markers, 353
 micropropagation, 354–355
 RAPD markers, 353
 virus elimination, 355
fire blight, 477, 480, 492, 495, 546, 555, 557
flavonoids, 571, 592
flowering
 juvenility, 415
 promotion, 4–5
 mango, 53
 pineapple, 160
regulation
 apple, 483–484
 kiwifruit, 8
 papaya, 180–181
 pineapple, 160
Fragaria see strawberry
Fusarium wilt, 367, 378

Garcinia mangostana see mangosteen
gene cloning see cloning, gene
gene silencing, 318
genebanks
 GenBank, 314(tab)
 International Coconut genebank, 100, 102, 103(tab)
genetic mapping
 almond, 521
 apple, 487
 avocado, 331
 banana and plantain, 370
 blueberry, 232–233
 cacao, 645–646, 647(tab)
 cane fruit, 572–573
 cherry, 521
 chestnut, 272
 citrus, 594–595
 coconut palm, 94
 European olive, 408–409
 grape, 678
 oil palm, 118–119
 papaya, 182, 183
 passionfruit, 441, 442(fig)
 peach, 521
 pear, 550
 strawberry, 461
genetic modification, xxiii–xxiv
genetic transformation
 Annona spp., 81
 apple, 492–496
 avocado, 339–340

- genetic transformation *continued*
 banana and plantain, 378–381
 blueberry, 238–239
 cacao, 659–660
 cane fruit, 576–577
 chestnut, 284
 citrus, 605–611
 cranberry, 252–256
 European olive, 415–418
 grape, 694–696
 selected literature, 688–689(tab)
 guava, 398
 kiwifruit (*Actinidia* spp.), 18–19, 20(tab)
 litchi and longan, 633
 mango, 54–55
 mangosteen, 217–218
 mulberry, 357
 oil palm, 130–134
 papaya, 186–195
 passionfruit, 448–449
 pear and quince, 555–557
 pecan, 303–34
 pineapple, 166–167
 Prunus spp., 528–531
 strawberry, 465–468
 walnut, 317–318
- genomics, xxi–xxii
- genotype: in grape embryogenic induction, 682
- Germplasm Resource Information Network, 476
- gibberellins, 483
- Global *Musa* Genomics Consortium, 369
- globulins, oil palm, 126–127
- grape
 botany and history, 672–673
 breeding and genetics, 673–677
 clonal selection, 675
 DNA markers, 677–678
 economic importance, 673
 embryo rescue, 676–677
 gene cloning, 677–678
 genetic mapping, 678
 genetic transformation, 694–696
 selected literature, 688–689(tab)
 germplasm conservation, 695–697
 haploid recovery, 691
 inbreeding depression, 674
 isoenzyme polymorphism analysis, 678–679
 micropropagation, 679–670
 mutagenesis and somaclonal variation, 691–693
 organogenesis, 690–691
 pathogen elimination, 680
 pests and diseases, 674, 675–676, 693, 695–696
 pollination, 673
 protoplast isolation and culture, 691
 rootstocks, 674
 scions, 674–677
 seedlessness, 675, 676–677
 somatic embryogenesis, 696–697
 selected literature, 680–687, 682–684(tab), 690
 somatic hybridization, 693–694
 taxonomic revision, 679
 viruses, 695
- grapefruit *see* citrus
- grey mould, 576, 577
- guava
 botany and history, 394
 dwarfism, 396
 economic and nutritional importance, 394–395
 germplasm preservation, 398
 haploid recovery, 398
 micropropagation, 396–397
 organogenesis, 397
 pests and diseases, 395–396
 rootstocks, 395–396
 scions, 395
 somatic embryogenesis, 397–398, 399(fig.)
 transformation, genetic, 398
 yield, 396
 yields, 396
- haploid recovery
 Annona spp., 80
 apple, 490–491
 cacao, 658
 cashew, 36
 citrus, 599
 coconut palm, 98
 date palm, 151
 European olive, 414
 grape, 691
 guava, 398
 kiwifruit, 12
 litchi and longan, 632
 mulberry, 356–357
 papaya, 185
 pineapple, 165
 Prunus spp., 526
 strawberry, 464
- herbicides, resistance to, 186, 189(tab), 195, 255–256, 468
- history *see* botany and history
- hybridization, somatic
 avocado, 338–339
 banana and plantain, 379
 citrus, 603–605
 grape, 693–694
 kiwifruit, 17–18
 litchi and longan, 634
 mangosteen, 217
 papaya, 186
 passionfruit, 446–448
 pear-cherry, 554–555
 Prunus spp., 528

- hyperhydricity, 51
hypoglycaemia, induction of, 350
hypomethylation, DNA, 128–129
- inbreeding depression, 674
indexing techniques: coconut germplasm, 101(tab)
ink disease, 268–270
insects *see* pests and diseases
International Atomic Energy Agency, 151
International Chestnut Commission, 269
International Coconut genebank, 100, 102, 103(tab)
invertase, 180
iron, dietary, 223
iron chlorosis, 545
isoenzyme analysis
 Annona spp., 77
 apple, 484
 blueberry, 229
 cane fruit, 571
 chestnut, 271(tab), 272
 citrus, 587, 588
 coconut palm, 94
 date palm, 146–147
 fig, 353
 grape, 678–679
 kiwifruit, 3
 mango, 43
 mangosteen, 212
 mulberry, 353
 passionfruit, 440
 pear and quince, 547, 549(tab)
 pineapple, 161
ISSR (inter-simple sequence repeat) markers *see*
 under DNA markers
- jacalin, 352
jackfruit *see* *Artocarpus* spp.
Juglandaceae, 297
 see also pecan; walnut
Juglans regia *see* walnut
- K segments, 225, 226, 227
kiwifruit
 adverse effects, 4
 botany and history, 2–3
 cryopreservation, 19
 functional genomics, 7–8
 gene cloning, 6
 genetic analysis, 3
 genetic transformation, 18–19, 20(tab)
 haploid recovery, 12
 marker-assisted selection, 6–7
 micrografting, 10
 micropropagation, 9
 mutagenesis and somaclonal variation, 14,
 16–17
 nutritional and medicinal importance, 3–4
 organogenesis, 10–12
 pests, 5
 protoplasts
 culture and isolation, 12–14, 15(fig.)
 fusion, 17–18
 rootstocks
 breeding accomplishments, 4–5
 breeding objectives, 4
 scions, 5–6
 somatic embryogenesis, 10
 somatic hybridization, 17–18
 virus elimination, 10
- Laie Gold (papaya), 191–192
Lauraceae, 325
 see also avocado
lemon *see* citrus
lignification, 11
lime *see* citrus
linkage mapping *see* genetic mapping
litchi
 botany and history, 628
 breeding and genetics, 629
 DNA markers, 630
 fruit quality, 629
 genetic transformation, 633
 haploid recovery, 632
 micropropagation, 630
 pests and diseases, 629
 protoplast isolation and culture, 632–633
 somatic embryogenesis, 631–632
 somatic hybridization, 634
longan
 botany and history, 628
 breeding and genetics, 629
 cryopreservation, 634
 fruit quality, 629
 genetic transformation, 633
 haploid recovery, 632
 protoplast isolation and culture, 632
 somatic embryogenesis, 630–631
 somatic hybridization, 634
lychee *see* litchi
- Malus* × *domestica* *see* apple
mandarin *see* citrus
Mangifera indica *see* mango
mango
 alternate oxidase, 46
 anthracnose, 53
 aroma and flavour, 46
 botany and history, 40–41

- mango *continued*
- cell wall synthesis and turnover, 46–47
 - conventional breeding, 41–42
 - cryopreservation, 56
 - developmental arrest and desiccation, 55–56
 - DNA markers, 43–44
 - economic importance, 41
 - functional genomics, 47
 - gene cloning, 44–47
 - genetic transformation, 54–55
 - mutagenesis and somaclonal variation, 53–54
 - organogenesis, 52
 - promotion of flowering, 5
 - protein markers, 43
 - protoplast isolation and culture, 52–53
 - resistance, 53–54
 - ripening, 44–47, 55
 - rootstocks, 42
 - scions, 42–43
 - somatic embryogenesis
 - germination, 51–52
 - induction, 47–49
 - maintenance, 49–50
 - maturation, 50–51
- mangosteen
- botany and history, 211
 - breeding and genetics, 212
 - economic importance, 211–212
 - genetic transformation, 217–218
 - micropropagation, 214
 - molecular markers, 212, 214
 - morphological characteristics, 213(tab)
 - mutagenesis and somaclonal variation, 216–217
 - organogenesis, 214–215
 - protoplast isolation and culture, 215–216
 - somatic embryogenesis, 214
 - somatic hybridization, 217
- mantled* abnormality (oil palm), 120, 128–130
- mapping, genetic *see* genetic mapping
- marker-assisted selection *see* selection, marker-assisted
- markers *see* DNA markers; isoenzyme analysis
- markers, polyphenol, 94
- micrografting
- apple, 489
 - avocado, 335
 - cashew, 34
 - citrus, 596–597
 - kiwifruit, 10
 - pear, 551
 - pistachio, 65
 - Prunus* spp., 523
- microprojectile bombardment *see* biolistics
- micropropagation
- Annona* spp., 78–79, 82–84(tab)
 - apple, 488–489
 - Artocarpus* spp., 355
 - avocado, 331–335
 - banana and plantain, 371–374, 381
 - blueberry, 234–236
 - cacao, 650–651
 - cane fruit, 573
 - cashew, 33–34
 - chestnut, 272–276
 - citrus, 596
 - comparison with grafts and cuttings, 9
 - cranberry, 249–250, 251(fig.)
 - date palm, 147
 - European olive, 411–412, 413(tab)
 - fig, 354–355
 - grape, 679–670
 - guava, 396–397
 - kiwifruit, 9
 - litchi and rambutan, 630
 - mangosteen, 214
 - mulberry, 352–354
 - papaya, 183–184
 - passionfruit, 441, 443
 - pear, 552(tab)
 - pear and quince, 550–551, 552(tab), 553
 - pecan, 300
 - pineapple, 162–163
 - pistachio, 65, 66(tab)
 - Prunus* spp., 523
 - strawberry, 462, 464–465
 - walnut, 316
- microsatellites *see* DNA markers
- mildew, powdery, 32
- Moraceae*, 349
- see also* *Artocarpus* spp.; fig; mulberry
- Morus* spp. *see* mulberry
- mucilage and protoplast purification, 14
- mulberry
- botany and history, 350
 - breeding and genetics, 352
 - cryopreservation, 358
 - gene cloning, 352–353
 - genetic transformation, 357
 - haploid recovery, 356–357
 - isoenzyme markers, 353
 - medical properties, 350
 - micropropagation, 352–354
 - mutagenesis and somaclonal variation, 357
 - organogenesis, 355–356
 - protoplast isolation and culture, 357
 - and silk industry, 350, 352–353
 - somatic embryogenesis, 356
 - triploid recovery, 357
- multi-autotransformation vectors, 418
- Musa* spp. *see* banana and plantain
- Musaceae*, 365
- apple, 491–492
 - avocado, 338

- banana and plantain, 376–378
- blueberry, 237–238
- cacao, 658–659
- cane fruit, 574–576
- citrus, 601–602
- date palm, 151
- European olive, 415
- grape, 691–693
- kiwifruit, 14, 16–17
- mango, 53–54
- mangosteen, 216–217
- mulberry, 357
- oil palm, 128–130
- papaya, 186
- pear and quince, 553–554
- pecan, 302–303
- pineapple, 165–166
- Prunus* spp., 527–528
- strawberry, 464–465
- see also* banana and plantain
 - mutagenesis and somaclonal variation
- mycorrhiza: inoculation of rootstocks, 5
- Myrtaceae*, 393
- see also* guava
- National Clonal Germplasm Repository (NCGR),
 - USA, 257
- nectarine *see* peach and nectarine
- nematodes, 310, 522–523
- nucellar embryony, 586
- oil palm
 - acquisition of tolerance to dessication, 126
 - biodegradable thermoplastics, 133–134
 - botany and history, 113
 - breeding, 114
 - cryopreservation, 134
 - DNA markers, 114–115
 - economic importance, 113
 - functional genomics, 120
 - gene cloning, 120–123
 - genetic mapping, 118–119
 - genetic transformation
 - accomplishments, 132–134
 - objectives, 130–131
 - protocol, 131–132
 - mantled* abnormality, 120, 128–130
 - marker-assisted selection, 115–118
 - mutagenesis and somaclonal variation, 128–130
 - regeneration
 - development and maturation, 124–127
 - plant recovery, 127–128
 - somatic embryogenesis, 123–124
 - storage proteins, 126–127
 - transgenic plant, 132(fig.)
- oils
 - coconut, 92
 - olive, 405, 406, 415
 - palm, 113
 - walnut, 314, 315
- Olea europaea* *see* olive, European
- Oleaceae*, 403
- see also* olive, European
- oleic acid, 131
- olive, European
 - botany and history, 404–405
 - cold tolerance, 416
 - cryopreservation, 418
 - cultivar identification, 410–411
 - economic importance, 405
 - gene cloning, 406–408
 - genetic transformation, 415–418
 - growth habit modification, 417
 - haploid recovery, 414
 - juvenility, 415
 - linkage mapping, 408–409
 - marker-assisted selection, 408–409
 - micropropagation, 411–412, 413(tab)
 - molecular markers, 409–411
 - multi-autotransformation vectors, 418
 - mutation induction, 415
 - oil, 405, 406, 415
 - organogenesis, 414
 - parthenocarpy, 415
 - pests and diseases, 405, 416, 417–418
 - polyploids, 415
 - protoplast isolation and culture, 415
 - relationships between cultivated and wild, 410
 - ripening, 416
 - rooting efficiency, 417
 - rootstocks, 405
 - scions, 405–406
 - somatic embryogenesis, 412, 414
 - taxonomic revision, 409–410
 - tree architecture, 416
 - virus elimination, 412
- omega-3 fatty acids, 314–315
- orange *see* citrus
- organogenesis
 - Annona* spp., 79–80, 82–84(tab)
 - apple, 490
 - Artocarpus* spp., 356
 - blueberry, 236–237
 - cane fruit, 573–574
 - carambola, 432
 - cashew, 34–35
 - chestnut, 281–284
 - citrus, 599
 - cranberry, 250–251
 - date palm, 150–151
 - European olive, 414
 - grape, 690–691

- organogenesis *continued*
 guava, 397
 kiwifruit, 10–12
 mango, 52
 mangosteen, 214–215
 mulberry, 355–356
 papaya, 185
 passionfruit, 444
 pineapple, 164–165
 pistachio, 67(tab), 68
 Prunus spp., 525–526
 strawberry, 463–464, 467(fig.)
oxalic acid, 431
Oxalidaceae, 429
 see also carambola
- palm, coconut *see* coconut palm
palm, date *see* date palm
palm, oil *see* oil palm
palmitic acid, 131
papain, 175, 181
papaya
 aluminium tolerance, 190(tab), 194
 arthropod resistance, 194
 BAC libraries, 462
 back-crossing and selection, 191–193
 botany and history, 174
 cold tolerance, 177–178, 181, 194–195
 cryopreservation, 195–196
 cystatins, 181
 DNA markers, 182–183
 economic importance, 175
 fine mapping and physical mapping, 183
 flower development, 180–181
 fruit quality, 179
 fungal/oomycete tolerance, 177, 178–179, 180, 193–194
 gene cloning, 179–181
 genetic transformation
 accomplishments, 190–195
 breeding objectives, 186
 protocol, 186–187, 190
 research groups, 188–190(tab)
 genetics, 175–176
 genomics, 181–183
 haploid recovery, 185
 Hawaii, 191
 herbicide resistance, 186, 189(tab), 194
 linkage maps, 182
 marker-assisted selection, 183
 micropropagation, 183–184
 mutagenesis and somaclonal variation, 186
 organogenesis, 185
 pests and diseases, 176–177, 178–179, 180, 188–189(tab), 190–191, 192–194
 pollination, 176
 protoplast isolation and culture, 185
 ripening, 179–180, 189(tab), 194–195
 somatic embryogenesis, 184–185
 somatic hybridization, 186
 virus resistance, 176–177, 178, 188, 190–191, 192–193
parthenocarp, 415
parthenogenesis, 12
Passifloraceae, 435
 see also passionfruit
passionfruit
 botany and history, 436, 438
 breeding objectives, 439
 commercial species, 437–438(tab)
 economic importance, 438
 gene isolation and mapping, 441, 442(fig)
 genetic transformation, 448–449
 genetics, 438
 markers, 440
 micropropagation, 441, 443
 organogenesis, 444
 pests and diseases, 439
 pollen viability, 447(tab)
 protoplast isolation and culture, 445–446
 rootstocks, 440
 scions, 439–440
 somatic embryogenesis, 443–444
 somatic hybridization, 446–448
 triploid recovery, 444–445
 virus indexing, 443
 woodiness virus, 439, 449
peach and nectarine
 allergenicity, 520
 botany and history, 512
 cab promoter, 520
 cultivar identification, 521–522
 dehydrins, 520
 economic and nutritional importance, 513–514
 genetic transformation, 528, 529(tab)
 haploid recovery, 526
 linkage mapping, 521
 micropropagation, 523
 model for *Prunus* genome, 518
 mutagenesis and somaclonal variation, 527–528
 organogenesis, 525
 pests and diseases, 527–528
 ripening, 518–519
 rootstocks, 516
 scions, 516–517
 somatic embryogenesis, 524
 virus elimination, 523
pear
 botany and history, 543
 cold tolerance, 544, 546
 cryopreservation, 557–558
 cultivars and consumer familiarity, 558
 DNA markers, 547, 549(tab), 550

- dwarfism, 557
- economic importance, 543
- fruit quality, 545
- gene cloning, 546–547
- genetic transformation, 555–557
- isoenzyme polymorphism analysis, 547, 549(tab)
- linkage mapping, 550
- micropropagation, 550–551, 552(tab), 553
- mutagenesis and somaclonal variation, 553–554
- NCBI-ENTREZ entries, 548
- pests and diseases, 544, 546, 555, 557
- plant defence genes, 546–547
- protoplast isolation and culture, 553
- ripening, 547
- rootstocks, 544–545
- scions, 545–546
- self-incompatibility, 547
- somatic hybridization, 554–555
- virus elimination by micrografting, 551
- pecan
 - botany and history, 298
 - breeding and genetics, 299–300
 - characteristics, 299
 - cryopreservation, 304
 - economic importance, 298–299
 - genetic transformation, 303–34
 - micropropagation, 300
 - molecular genetics, 300
 - mutagenesis and somaclonal variation, 302–303
 - pests and diseases, 299, 303
 - somatic embryogenesis, 300–302
- Persea americana* *see* avocado
- pests and diseases
 - Annona* spp., 75, 76
 - apple, 477, 479–480, 487–488, 492, 493–494
 - avocado, 328, 329, 338–339, 341
 - banana and plantain, 367, 368
 - bayoud* disease, 145–146, 151
 - blueberry, 224
 - cacao, 642–643, 647(tab), 648–649
 - cane fruit, 576, 577
 - cashew, 31, 32, 33
 - chestnut, 268–270
 - citrus, 595–596, 605
 - coconut palm, 100, 101(tab), 102(tab)
 - cranberry, 253–254
 - date palm, 145–146
 - European olive, 405, 416, 417–418
 - grape, 674, 675–676, 693, 695–696
 - guava, 395–396
 - kiwifruit, 5
 - litchi and longan, 629
 - mango, 53–54
 - papaya, 176–177, 178–179, 180, 188–189(tab), 190–191, 192–194
 - passionfruit, 439
 - peach, 527–528
 - pear and quince, 544, 546, 555, 557
 - pecan, 299, 303
 - pineapple, 159, 166
 - pistachio, 63–64
 - Prunus* spp., 522–523
 - strawberry, 457, 465, 466
 - walnut, 309–311, 313, 317–318
 - see also* viruses
- phenylpropanoid pathway, 315
- Phoenix dactylifera* *see* date palm
- Phytophthora* rot
 - apple, 477
 - avocado, 328, 338–339, 341
 - cacao, 642, 647(tab), 648–649
 - chestnut, 268–270
 - citrus, 605
 - papaya, 178–179, 194
 - walnut, 309–310
- Pierce's disease, 693
- pigmentation, fruit, 248
- pineapple
 - biolistics, 166
 - blackheart, 160–161
 - botany and history, 158–159
 - breeding and genetics, 159–160
 - DNA markers, 161–162
 - economic importance, 159
 - flowering, 160
 - gene cloning, 160–161
 - genetic transformation, 166–167
 - germplasm conservation, 167
 - haploid recovery, 165
 - micropropagation, 162–163
 - mutagenesis and somaclonal variation, 165–166
 - organogenesis, 164–165
 - pests and diseases, 159, 166
 - protoplast isolation and culture, 165
 - ripening, 160
 - somatic embryogenesis, 164
- Pineapple Research Institute, Hawaii, 159–160
- pistachio
 - botany and history, 62–63
 - diseases, 63–64
 - economic importance, 63
 - genetic manipulation, 68–69
 - germplasm storage, 69
 - micropropagation, 65, 66(tab)
 - organogenesis, 67(tab), 68
 - rootstocks, 63–64
 - scions, 64
 - somatic embryogenesis, 67–68
- Pistacia vera* *see* pistachio
- Plan de Mejora y Regeneración del Castaño, 269–270
- plantains *see* banana and plantain
- plasmids and oil palm transformation, 132(tab)

- plastics, biodegradable, 133–134
- plum
- botany and history, 512–513
 - cryopreservation, 531
 - economic and nutritional importance, 514
 - genetic transformation, 529(tab), 530–531
 - haploid recovery, 526
 - micropropagation, 523
 - organogenesis, 525–526
 - rootstocks, 516
 - scions, 517–518
- pollination
- Annona* spp., 75
 - coconut palm, 93, 99–100
 - grape, 673
 - papaya, 176
 - passionfruit somatic hybrid pollen viability, 447(tab)
- polyphenol markers, 94
- polyphenol oxidase, apple, 483
- powdery mildew, 32
- protein markers *see* isoenzyme analysis
- proteins, storage, 126–127
- protoplasts
- fusion, 17–18
 - isolation and culture
 - Annona* spp., 81, 83(tab)
 - apple, 491
 - avocado, 337–338
 - banana and plantain, 377
 - cacao, 658
 - cane fruit, 574
 - citrus, 601
 - coconut palm, 98
 - European olive, 415
 - grape, 691
 - kiwifruit, 12–14, 15(fig.)
 - litchi and longan, 632–633
 - mango, 52–53
 - mangosteen, 215–216
 - mulberry, 357
 - papaya, 185
 - passionfruit, 445–446
 - pear, 553
 - pineapple, 165
 - Prunus* spp., 527–528
 - strawberry, 464
- Prunus* spp.
- botany and history, 513
 - cryopreservation, 531
 - economic and nutritional importance, 513–514
 - gene cloning and functional genomics, 518–520
 - genetic transformation, 528–531
 - genome, 514, 515 (fig.)
 - haploid recovery, 526
 - incompatibility (self and cross), 519
 - marker-assisted selection, 520–523
 - micropropagation, 523
 - mutagenesis and somaclonal variation, 527–528
 - organogenesis, 525–526
 - pests and diseases, 522–523, 527–528
 - protoplast isolation and culture, 526–527, 527–528
 - rootstocks, 514, 516
 - scions, 516–518
 - somatic embryogenesis, 524–525
 - somatic hybridization, 528
 - virus elimination, 523
- Psidium guajava* *see* guava
- pummelo *see* citrus
- Pyrus* spp. *see* pear
- QTL analysis
- apple, 481, 487
 - cacao, 646, 648, 649–650
 - citrus, 594–595
 - coconut palm, 94
 - oil palm, 115, 118–119
 - papaya, 182
 - peach, 521
- quality
- Annona* spp., 75–76, 76–77
 - apple, 479, 481, 487
 - avocado, 328
 - blueberry, 224, 225
 - cashew, 32
 - chestnut, 269
 - fruit
 - mango, 46
 - impact of biotechnology, xx
 - litchi and longan, 629
 - mangosteen, 212
 - papaya, 179
 - pear, 545
 - walnut, 312–313, 314–315
- quince
- cold tolerance, 544, 545
 - DNA markers, 547, 549(tab)
 - genetic transformation, 555
 - iron chlorosis, 545
 - isoenzyme polymorphism analysis, 547, 549(tab)
 - micropropagation, 550–551, 552(tab), 553
 - mutagenesis and somaclonal variation, 553–554
 - NCBI-ENTREZ entries, 548
 - rootstocks, 544–545
- Rainbow (papaya), 190–191, 192
- rambutan: micropropagation, 630
- RAPD markers *see* DNA markers
- raspberry *see* cane fruit
- raspberry bushy dwarf virus, 570, 576–577

- resistance, disease and pest *see* pests and diseases
- RFLP (restriction fragment length polymorphism)
analysis *see* DNA markers
- ricinoleic acid, 133
- ripening
 Annona spp., 76, 81
 apple, 482
 avocado, 328–329, 330, 340
 banana and plantain, 379
 cane fruit, 576
 citrus, 592
 European olive, 416
 kiwifruit, 7–8
 mango, 44–47, 55
 papaya, 179–180, 189(tab), 194–195
 peach and nectarine, 518–519
 pear, 547
 pineapple, 160
 strawberry, 458–459, 466–467
- RITA (récepteur à immersion temporaire
aéropulse) system, 656, 657 (fig.)
- rooting
 auxin metabolism, 333–334
 increased efficiency, 417
- rootstocks
 apple, 477–478
 avocado, 327–328
 carambola, 431
 citrus, 590
 European olive, 405
 and flower promotion, 4–5
 grape, 674
 guava, 395–396
 mangosteen, 212
 mycorrhizal inoculation, 5
 passionfruit, 440
 pear and quince, 544–545
 Prunus spp., 514, 516
- Rosaceae, 455
 see also almond; apple; apricot; cane fruit;
 cherry; peach and nectarine; pear; plum;
 Prunus spp.; quince; strawberry
- rosy leaf-curling aphid, 480
- Rubus* spp. *see* cane fruit
- Rutaceae, 583
 see also citrus
- safety, food, xx
- salinity, tolerance to, 328
- Sapindaceae, 627
 see also litchi; longan
- satsuma *see* citrus
- Saurauia*, 1
- SCAR markers *see* DNA markers
- scions
 Annona spp., 75–77
 apple, 479–482
 avocado, 328–329
 carambola, 431
 cashew, 31–33
 citrus, 591
 European olive, 405–406
 grape, 674–677
 guava, 395
 kiwifruit, 5–6
 litchi and longan, 629
 mango, 42–43
 passionfruit, 439–440
 pear, 545–546
 pistachio, 64
 Prunus spp., 516–518
 walnut, 311–313
seed: used as currency, 640
seedlessness, 675, 676–677
selection, marker-assisted
 apple, 484–488
 avocado, 331
 blueberry, 228
 cacao, 645–650
 citrus, 594
 coconut palm, 94
 European olive, 408–409
 kiwifruit, 6–7
 mango, 44
 oil palm, 115–118
 papaya, 183
 pineapple, 161–162
 Prunus spp., 520–523
 strawberry, 459–461
 walnut, 315
sensory evaluation, 481
Serr-Forde walnut breeding programme, 312
sex: in grape embryogenic induction, 681
shoots, induction of *see* organogenesis
silencing, gene, 318
silk and silkworms, 350, 352–353
somaclonal variation *see* mutagenesis and
 somaclonal variation
somatic embryogenesis *see* embryogenesis,
 somatic
somatic hybridization *see* hybridization, somatic
sorbitol: synthesis, 483
sour cherry *see* cherry
soursop *see* *Annona* spp.
Spain, 269–270
star fruit *see* carambola
stearic acid, 131, 133
Sterculiaceae, 637
 see also cacao
storage proteins, 126–127
strawberry
 botany and history, 456
 breeding objectives and accomplishments,
 457–458

- strawberry *continued*
 cold tolerance, 468
 cryopreservation, 468
 cultivar identification, 460
 economic importance, 457
 fruit flavour, 461
 functional genomics, 461–462
 gene cloning, 458–459
 genetic mapping, 461
 genetic relationships, 460
 genetic transformation, 465–468
 haploid recovery, 464
 herbicide resistance, 468
 marker-assisted selection, 459–461
 micropropagation, 462, 464–465
 mutagenesis and somaclonal variation, 464–465
 organogenesis, 463–464, 467(fig.)
 pests and diseases, 457, 465, 466
 protoplast isolation and culture, 464
 ripening, 458–459, 466–467
 somatic embryogenesis, 463
 virus elimination, 463
 sugar apple *see* *Annona* spp.
 SunUp (papaya), 190–191
 superficial scald, 483
- tangerine *see* citrus
- taxonomy
 citrus, 585–590
 European olive, 409–410
 grape, 679
- temporary immersion somatic embryogenesis
 system, 656, 657(fig)
- The American Chestnut Foundation, 270
- Theobroma cacao* *see* cacao
- thermoplastics, biodegradable, 133–134
- thermotherapy: virus elimination, 573
- thioesterases, 121
- tissue culture: general remarks, xxi
- transformation, genetic *see* genetic transformation
- transgenes, restricting expression of, 495
- tree architecture, 212
Annona spp., 76
 apple, 480
 avocado, 329
 European olive, 416
 walnut, 318
- triploid recovery
Annona spp., 80–81
 cane fruit, 574
 citrus, 599–601
 kiwifruit, 12
 mulberry, 357
 passionfruit, 444–445
 walnut, 317
- Vaccinium* spp. *see* blueberry; cranberry
- variation, somaclonal *see* mutagenesis and somaclonal variation
- viruses
 avocado, 335
 banana and plantain, 374–375
 banana streak badnavirus, 371
 cacao, 642, 659, 660
 cane fruit, 576–577
 chestnut, 310
 citrus, 595
 elimination
 apple, 489
 avocado, 335
 banana, 374–375
 cane fruit, 573
 citrus, 596–597
 European olive, 412
 fig, 355
 grape, 680
 kiwifruit, 10
 passionfruit, 443
 pear, 551
 Prunus spp., 523
 strawberry, 463
 grape, 695
 papaya, 176–177, 178, 188, 190–191, 192–193
 passionfruit, 443, 449
 walnut, 310, 313
- Vitaceae*, 671
see also grape
- Vitis* spp. *see* grape
- walnut
 botany and history, 307–308
 breeding and genetics, 308
 rootstocks, 309–311
 scions, 311–313
 cryopreservation, 318
 economic importance, 308
 functional genomics, 315
 GenBank entries, 314(tab)
 gene cloning, 314–315
 genetic manipulation, 317–318
 hybrids, 398(fig.)
 marker-assisted selection, 315
 medicinal properties, 315
 micropropagation, 316
 nut quality, 312–313, 314–315
 pedigree of major cultivars, 311(fig.)
 pests and diseases, 309–311, 313, 317–318
 phenology, 313
 somatic embryogenesis, 316–317
 triploid recovery, 317
 vigour, 309
 yield, 312

witches' broom, 642–643
woodiness virus, 439, 449
woolly apple aphid, 477, 480

cacao, 643, 646, 648
guava, 396
walnut, 312

xylanase, 180

ZESPRI™ Gold, 4

yield